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PHENANTHRIDINIUM 1553 AS A PREVENTIVE AGAINST ANIMAL TRYPANOSOMIASIS IN THE GOLD COAST

By

J. L. Stewart.

In a previous paper (Stewart, 1946) it has been shown that Phenanthridinium 1553 is effective as a curative of trypanosomiasis in the Gold Coast except that caused by *T. brucei* and that the drug does not cause toxic symptoms even when its use is pushed. But it is equally important that a preventive drug shall be available as most of the meat animals travel on foot across the Colony from French West Africa, a journey of about three weeks, during which time they frequently pass through areas infested by tsetse flies. It was thus decided to carry out experiments to ascertain the possible value of Phenanthridinium 1553 as a preventive.

METHODS

Cattle

The cattle used were, with the exception of nine Zebu cows, young native animals of the West African shorthorn unhumped type, although it will be noted that in several of the experiments cattle with a high Zebu make-up were used.

In order to check that *T. vivax* transmits easily, six animals were inoculated subcutaneously with 10 ml. of citrated blood from a naturally infected case. With one exception, all became infected 12

Table I

Prophylactic Effect of Phenanthridinium 1553 against *T. vivax* Infection in Cattle

Number of animals used	*Interval between drug treatment and test dose.* *Days*	*Trypanosomes in blood.* *Days*
3 treated 3 controls	7	— — — 6, 8 and 21
3 treated 3 controls	11	— — * 15, 17 and 35
3 treated 3 controls	14	— — — 7, 7 and 9
3 treated 3 controls	21	— — — 16, 16 and 16
3 treated 3 controls	28	— — — 9, 15 and 18

* Died haemonchosis. — No T's in blood.

days later. In the exceptional case parasites were seen in the blood 43 days later. A further six animals were injected subcutaneously with 10 ml. of infected blood immediately after withdrawal and without citration. They became infected as follows: two after three days, one after ten, one after 14 and two after 39.

Throughout the experiments phenanthridinium was given in a dosage of 2 mg./kg. bodyweight by intravenous injection. The infected blood was injected subcutaneously in a dose of 10 ml. The results in relation to *T. vivax* and *T. congolense* are given in Tables I and II.

TABLE II

PROPHYLACTIC EFFECT OF PHENANTHRIDINIUM 1553 AGAINST *T. congolense* INFECTION IN CATTLE

Number of animals used	*Interval between drug treatment and test dose.* *Days*	*Trypanosomes in blood.* *Days*
3 treated 3 controls	7	— — — 7, 8 and 18
*3 treated *3 controls	8	— — — 13, 13 and 13
3 treated 3 controls	14	— — — 8, 9 and 11
*3 treated *2 controls	20	— — — 8 and 8
3 treated 3 controls	21	— — — 7, 8 and 9
*3 treated *3 controls	25	— — — 8, 8 and 8
3 treated 3 controls	28	— — — 7, 8 and 10

* These animals had been given a dose of *T. vivax* prior to *T. congolense* but had failed to react to the former.

A further experiment was carried out to test the efficiency of the drug as a prophylactic against trypanosome infection in Zebu cattle for these are generally considered to be more susceptible than the local West African shorthorn. White Fulani (Nigerian) cows were used. The results are given in Table III.

All the control animals, except one which died from haemonchosis, were subsequently treated with phenanthridinium successfully.

Pigs

A series of preliminary experiments were performed to ascertain the infectivity of *T. vivax* and *T. congolense* for pigs. The animals used were weaners. Results are given in Table IV.

TABLE III

PROPHYLACTIC EFFECT OF PHENANTHRIDINIUM 1553 AGAINST *T. vivax* AND *T. congolense* INFECTION IN ZEBU CATTLE

Number of animals used	*Species of trypanosome*	*Interval between drug treatment and test dose. Days*	*Trypanosomes in blood. Days*
3 treated 2 controls	*T. vivax*	14	— — — 16 and 16
2 treated 2 controls	*T. congolense*	14	— — 14 and 15

TABLE IV

ARTIFICIAL INFECTION OF PIGS WITH *T. vivax* AND *T. congolense*

Type of animal	*Number used*	*Species of trypanosome*	*Trypanosomes in blood*
Pure-bred British	5	*T. vivax*	One after 4 days, two after 5 and two after 7
Pure-bred British	5	*T. congolense*	One after 13 days, one after 19 and three after 20
Half-bred Native-British	6	*T. vivax* *T. congolense* mixed	*T. vivax.* Three after 5 days, one after 6 and two after 7 *T. congolense.* One after 21 days, one after 27 and four after 30

None of the pigs used in these experiments showed symptoms of infection; weight gains and blood counts were normal. The animals in Groups II and III were successfully treated with phenanthridinium two months after artificial infection. In Group I treatment was delayed and five months after infection parasites were still to be found in the blood although they were scanty.

Experiments were then performed to test the protective action of phenanthridinium against *T. vivax* and *T. congolense* in pigs. The drug was used in the same dosage as in cattle, *viz* : 2 mg./kg. but administration was by subcutaneous injection. Injection caused some local swelling but this was of no consequence. (Table V).

All the infected animals were subsequently cured by phenanthridinium.

A series of experiments were also carried out using *T. brucei.* They comprised the artificial infection of cattle, pigs, donkeys and dogs, and the use of phenanthridinium as a preventive and curative. It was found that pigs, donkeys and dogs could be readily infected but cattle not so easily. Phenanthridinium had neither protective nor curative effect against this organism.

TABLE V

PROPHYLACTIC EFFECT OF PHENANTHRIDINIUM 1553 AGAINST *T. vivax* AND *T. congolense* INFECTION IN PIGS

Type	*Number used*	*Species of trypanosome*	*Interval between drug treatment and test dose. Days*	*Trypanosomes in blood. Days*
Pure-bred	4 treated	*T. vivax*	9	— — — —
Pure-bred	4 treated 4 controls	*T. vivax*	28	— — — — 7, 8, 9 and 11
Pure-bred	4 treated	*T. congolense*	9	7, 22, 29 and 29
Pure-bred	5 treated	*T. congolense*	16	15, 16, 23 and 24

DISCUSSION

The results of this work indicate that Phenanthridinium 1553 will prevent infection of West African shorthorn cattle by *T. vivax* and *T. congolense* up to one month after administration. In a small experiment using Zebu cattle, similar protection was afforded for 14 days beyond which point work did not proceed as no more animals of this breed were available. No cattle which had been given the drug as a prophylactic showed parasites in the blood at any time after the test injection. All were examined for a period of two months and some for as long as four months.

The position with regard to the natural infection of pigs by *T. vivax* and *T. congolense* is vague. The writer has on one occasion found *T. congolense* in a pig blood-smear but never *T. vivax*. These experiments show that the pig can be readily infected artificially by both species and that phenanthridinium while acting as a preventive against *T. vivax* for periods up to 28 days, is ineffective in preventing *T. congolense* infection. Neither species causes apparent ill-health in the pig although the parasite may persist in the blood for long periods.

In all animals experimentally infected by *T. vivax* and *T. congolense*, phenanthridinium acted as a curative agent.

The logical outcome of this work is a large-scale field trial. This is not possible at present owing to the scanty trade between French West Africa and the Gold Coast.

SUMMARY

Phenanthridinium 1553 in a dosage of 2 mg./kg. by intravenous injection protected cattle against both *T. vivax* and *T. congolense* infection for periods up to one month after administration.

Pigs were similarly protected after the subcutaneous injection of the drug in the same dosage against artificial *T. vivax* infection but no protection against *T. congolense* occurred.

The drug had no protective or curative action against *T. brucei* infection in cattle, pigs, donkeys or dogs.

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THE DEMONSTRATION OF AN AGGLUTININ TO *TRICHOMONAS FOETUS* IN THE VAGINAL DISCHARGE OF INFECTED HEIFERS

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INTRODUCTION

The diagnosis of bovine trichomoniasis has been a major problem since the recognition of *Trichomonas foetus* as a specific cause of infertility and abortion. Of the many methods investigated, the direct microscopical observation of the motile trichomonad in discharges collected from the vagina still remains the most satisfactory for positive diagnosis. Several workers during the course of such examinations, have noted that initial infection of the female is succeeded by a period during which trichomonads cannot be found. There follows a period when organisms can be found daily. Later they appear at intervals only and eventually disappear. The reason for these variations has remained obscure ; nor has any reason been found for the great variation in the duration of motility of the organisms. In some cases it is well maintained in discharges for many hours, while in others it ceases within an hour or two of the collection of the material. This fact has made early examination essential. In an endeavour to explain these observations. it was decided to study the reaction of the living trichomonad under the influence of discharges collected at frequent intervals from the vagina of heifers infected by coitus. As already reported (Pierce, 1946b), the results of these experiments have shown that an agglutinin to *T. foetus* develops in vaginal discharges of infected animals.

MATERIALS AND METHODS

Infection of animals and collection of samples for mucus tests.

Infection was initiated by allowing an infected bull (807) to serve four virgin heifers (710, 703, 663, 652). Two uninfected heifers (705 and 660), were also kept as controls. At frequent intervals mucus samples from both groups were aspirated from the vagina into sterilised glass pipettes, a separate pipette being used for each animal. A small portion of mucus was examined microscopically for *T. foetus* and the remainder weighed, resolved in a Griffiths tube to a 1 : 5 dilution with sterile 0·85 per cent. saline * containing 2 per cent. glucose, sealed in test tubes, and stored in the refrigerator.

Strains of T. foetus used in tests and cultural technique. The following strains of *T. foetus* were used to make test suspensions:

(*a*) " *Belfast* "—isolated by Kerr and Robertson in N. Ireland.

* All saline was prepared from glass distilled water.

(*b*) " Manley "—isolated by Mahmoud (1944), from an outbreak of trichomoniasis in Cheshire.

(*c*) " Stafford "—isolated by the author from the discharge of heifer 710 infected by coitus with bull 807 during these experiments. The method of isolation was a modification of that devised by Mahmoud (1944).

The three strains were maintained in glucose broth on an inspissated serum slope (Kerr and Robertson, 1943). Although no bacteria were demonstrated by bacteriological and staining techniques during the time of experiment no claim is made that the strains were strictly sterile at all times during their *in vitro* cultivation. The modified Mahmoud technique was also used in the occasional cultural examination of discharges that were negative to *T. foetus* by direct observation. Kerr and Robertson (1945) have found the Manley and Belfast strains suitable for agglutination work, and also to show a considerable degree of individual specificity.

Technique of the Mucus Test. The technique used to show the presence of agglutinins in mucus samples was developed from that devised by Fleming (1922) for the demonstration of lysozyme and that of Robertson (1941) for the demonstration of blood agglutinins to *T. foetus*. Mucus samples already resolved were diluted further with sterile glucose saline in $6'' \times \frac{5}{8}''$ test tubes, to give the following dilutions—1/5, 1/10, 1/20, 1/40, with a final volume of 2 c.c. in each tube. A further series of tubes, each containing 2 c.c. of nutrient agar, was melted and placed in a water bath at 60° C. Two c.c. of nutrient agar were then thoroughly mixed with each dilution of mucus and poured into small petri dishes ($1\frac{1}{2}''$ diam.) which were set aside and allowed to solidify.

A suspension of trichomonads prepared in a manner similar to that used in the blood agglutination test of Robertson (1941) and Kerr and Robertson (1941) was then pipetted on to the surface of each plate in 1·5 c.c. quantities. The plates after incubation at 37° C. for one and a half hours, were left at room temperature away from direct light for one hour. Microscopical observations under the 2/3" objective was then made for assessment of agglutination of the trichomonad suspension.

The system adopted for recording the degree of agglutination was that used by Kerr and Robertson (1941).

With each mucus test the following controls were included: —

(1) Agar, agar-saline, and trichomonad suspension to control all materials utilised in the test.

(2) Mucus-agar dilutions with mucus from uninfected heifers.

(3) Positive-serum-agar dilutions to control the specific agglutinability of the trichomonad suspension.

(4) Normal-serum-agar dilutions to control the nonspecific agglutinability. (See blood agglutination test below). Both 3 and 4, also control the slightly variable sensitivity of different trichomonad suspensions to agglutinin, and render it possible to compare one test with another. The serum was combined with agar to give final dilutions of 1/8, 1/16, 1/32, 1/64.

The Blood Agglutination Test. This test was carried out on serum from all the animals in these experiments, and was that used by Robertson (1941) and Kerr and Robertson (1941), already referred to. The method of assessing the agglutination was therefore similar in both tests, and the symbols used represent a similar degree of agglutination in each instance,

The use of the index*, rather than the titre, controls the slightly variable degree of agglutinability of suspensions prepared from *in vitro* cultures.

HISTORY OF ANIMALS USED IN INFECTION EXPERIMENTS

Bull 807 was born July, 1942, and became infected naturally in the autumn of 1943. It was bought by the Ministry of Agriculture in January, 1944 and in that year served on five occasions. In 1945 he served twice—heifers 710 and 652, and in 1946 twice also—heifers 703 and 663. Blood samples were consistently negative to agglutination tests for *T. foetus* and *Brucella abortus*. Since January, 1944 a high percentage of preputial washings were positive for trichomonads.

Heifers 710, 703 and 705, were bought as virgin heifers when one year eight months old. They were from a herd with clean breeding records and were negative to brucella and tuberculin tests. They were kept at grass on the Ministry's farm until required. Nos. 710 and 703 were served by infected bull 807 when three years and three and a quarter years old respectively. No. 705 was not served and was kept as a control.

Heifers 663, 652 and 660, were purchased when one and three quarter years old from a different source but with similar precautions. Heifers 663 and 652 when three and three quarter years and three and a half years old respectively were served by bull 807. Heifer 660, when three years eleven months old, was served by a clean bull. About one month before service blood samples were tested for brucella and trichomonas agglutinins, and specimens of vaginal mucus were examined for *T. foetus*, all with negative results.

Course of infection in the four infected heifers. Before tabulating the experimental results, the following brief account of the course of the infection in the heifers is given, and events noted with which the experimental data may be correlated.

(*a*) *Heifer* 652 (Sub-clinical infection). On the 27th day and again between the 36th and 42nd days after service, trichomonads were demonstrated in the vaginal discharge by direct examination. At no time was the discharge copious and it was consistently of the vaginal type. (Pierce, 1946a).

Pregnancy was diagnosed by rectal examination on the 60th day, and the heifer gave birth to a normal calf on the 280th day. At intervals throughout this period undiluted vaginal discharges were incubated at 37° C. in a glucose-broth-inspissated-serum medium to which penicillin had been added. Trichomonads were

* The numerator represents the total positive symbols in the 1/6, 1/12, 1/24, and 1/48 dilutions of the sera under test, and the denominator the degree of agglutination produced on the suspension by the normal agglutinins to be found in all bovine sera and to a less extent in all rabbit sera when diluted in a similar manner. Therefore, with each blood agglutination test, normal and known positive serum dilutions were included as controls. Saline suspension controls were used as a precaution against spontaneous agglutination.

demonstrated occasionally by this method up to the 52nd day after infection ; they were again demonstrated on the 132nd day, when after 48 hours culture several were observed having a rounded outline and showed very sluggish movement.

(*b*) *Heifer* 703 (Sub-acute infection). Trichomonads were first recovered nine days after service and then daily for 13 days, when the heifer returned to oestrus. During the three days following no organisms were recovered. (See Table VI). Organisms were constantly observed again in increasing numbers from the 25th to 44th day, being particularly numerous from the 38th to the 40th day when the discharge was watery and contained flakes of pus. On the 44th day the discharge was of the oestrous type and a slight trace of blood was noted on the 46th. The animal was presumed to have returned again to oestrus. Trichomonads could not be found by direct observation from the 45th to the 53rd day but were again observed by this method on the 54th and 55th days. Oestrus recurred a third time on the 64th day when the organism was demonstrated by cultural means. It was not again demonstrated in this animal.

(*c*) *Heifer* 663 (Sub-acute infection). The type of infection in this animal resembled heifer 703. Twelve days after service organisms were first recovered by direct observation, and the discharges remained positive until the 19th day when the animal returned to oestrus. On the 20th and 21st day there was some blood in the mucus and organisms were not recovered. They reappeared, however, on the following day and were recovered consistently up to the 31st day. Oestrus recurred on the 38th day but no organisms were again seen until they were finally demonstrated by cultural methods on the 56th day, the heifer returning to oestrus on the 58th day.

(*d*) *Heifer* 710 (Acute Infection). Trichomonads were first recovered fifteen days after service to the infected bull 807 and observed regularly from the 15th to 29th day. Rectal examination on the 60th day revealed a slight distension of the left uterine horn. From the 30th to 97th day trichomonads were recovered intermittently. Marked increase in numbers and increased motility during this period was noticed to be associated with the uterine type of discharge (Pierce, 1946a). During this period, discharges which had been found to be negative by direct examination frequently revealed organisms in culture. On these occasions they were usually very sluggish and round in outline. From the 97th to 114th day no further trichomonads were recovered by direct observation and the discharge had become scanty for the first time. On the 115th day a marked increase in discharge of the watery type, previously associated with the recovery of organisms in large numbers, revealed only one, moderately active, trichomonad. The heifer was slaughtered on the 121st day. No organisms were recovered from the vagina or uterus.

(*e*) *Two uninfected heifers* 660 *and* 705. Heifer 660 was served by a clean bull and became pregnant, while 705 was unmated and showed oestrus regularly throughout the period of observation. Frequent mucus samples and occasionally blood samples were collected from these animals.

EXPERIMENTAL DATA

Identification of the serological variety of the Stafford Strain.

Kerr and Robertson (1945) demonstrated two strains of *T. foetus* showing a considerable degree of individual antigenic specificity. The Stafford strain concerned in these infections was identified by agglutinating an active suspension of the Stafford strain with sera from a rabbit which had been immunized with a vaccine prepared from the Belfast strain and another with the Manley strain of *T. foetus*. The results set out in Table I show that the Stafford strain resembled more closely the Manley strain than the Belfast strain. As it has been shown (Kerr and Robertson, 1941 ; Morgan, 1943) that all strains are not suitable for agglutination work, it was decided to prepare most of the test suspensions with the Manley strain rather than with the newly isolated Stafford strain whose agglutinability might not be dependable.

TABLE I

THE AGGLUTINATION RESPONSE OF THE STAFFORD STRAIN OF *T. foetus* (HEIFER 710) TO SERA OF RABBITS ARTIFICIALLY IMMUNISED WITH THE MANLEY AND THE BELFAST STRAINS

	Agglutination indices. Serum from artificially immunised rabbits	
Strain used in suspension	*Belfast*	*Manley*
Belfast	15/5	7½/5
Manley	7½/5	17½/5
Stafford	8½/6	18½/6

Blood Agglutination Test on Infected Heifers.

This test was periodically carried out on sera from infected and control heifers. The only positive reactor was heifer 703 which had an agglutination index of 14½/10½ by the 63rd day of infection. Heifer 710 rose to the "above normal" index of 11½/8½ by the 102nd day and then began to wane. Heifer 652 became slightly above normal by the 140th day (index 11½/9½), while 663 and the uninfected heifers 705 and 660 remained normal throughout the experiment. That the positive blood titre developed by 703 was more marked against the Manley than against the Belfast strain, is shown in Table II. This confirms the closer antigenic relationship between the Manley and Stafford strains already recorded in Table I.

TABLE II

THE RELATIVE AGGLUTINATION OF THE MANLEY AND BELFAST STRAINS BY THE SERUM AGGLUTININS DEVELOPED BY HEIFER 703 TO THE STAFFORD STRAIN

Bovine serum	T. foetus *suspension*	*Agglutination results with various serum dilutions*				*Agglutination index*
		1/6	1/12	1/24	1/48	
703	Belfast	++++	++(+)	++	+(+)	$10/8\frac{1}{2}$
703	Manley	++++	+++(+)	+++	++(+)	$13/7\frac{1}{2}$
Serum controls Normal ...	Belfast	+++(+)	++(+)	+(+)	+	
Belfast positive*	Belfast	++++	++++	+++	++(+)	$13\frac{1}{2}/8\frac{1}{2}$
Manley positive*	„	++++	+++	++	+(+)	$10\frac{1}{2}/8\frac{1}{2}$
Normal ...	Manley	+++	++	+(+)	+	
Belfast positive*	„	+++(+)	++(+)	++	(+)	$8\frac{1}{2}/7\frac{1}{2}$
Manley positive*	„	++++	+++(+)	+++	+++	$13\frac{1}{2}/7\frac{1}{2}$

Saline suspension controls : Manley—Neg. Belfast—Neg.

* Positive serum controls :

Manley — from a heifer artificially immunised with a dead vaccine.

Belfast — from a heifer artificially infected with a live culture.

Mucus Tests

(*a*) *The demonstration of the mucus agglutinin.* In all four infected heifers, agglutinins to *T. foetus* were eventually demonstrated in the vaginal discharges. The first positive finding was in heifer 710 on the 65th day of infection, by which time agglutinins were appreciable in the 1/10 and 1/20 dilutions, with a trace in 1/40. In heifer 652 they were first detected on the 56th day, and in 703 on the 39th day. In 663, owing to the difficulty in obtaining sufficient mucus for the test agglutins were first detected on the 45th day when the titre was well developed. The highest titre was seen in heifer 710 which gave pre-agglutination and echelon formation in a 1/10 mucus-agar dilution. Heifer 703 which developed the sub-acute type of infection eventually gave echelon formation in a 1/10 dilution. Heifers 663 and 652 gave an average reading of $3\frac{1}{2}$ in the 1/10 dilution when the titre was fully developed. The controls 660 and 705 remained negative throughout. A typical series of mucus agglutinations is given in Table III.

Although slight fluctuations in the level of agglutination were observed from day to day in the same animal, a change in the nature of the mucus from a thick vaginal type to a more watery discharge often containing discrete flakes of pus, was accompanied by marked lowering in the agglutination level, which sometimes fell to zero. The latter type of discharge was thought to originate from the uterus, and in such discharges trichomonads were often numerous and active. A series of these observations is set out in Table IV which shows that the recovery of the motile trichomonad is associated with a decrease or the complete absence of the agglutinin and *vice-versa*. The continuous recovery of the organism

TABLE III

THE REACTION OF THE MANLEY STRAIN OF *T. foetus* TO MUCUS AGGLUTININS

Animal number	*Days after infecting service*	*Agglutination results. Mucus-agar dilutions*			
		1/10	1/20	1/40	1/80
		(E)			
703*	156 not pregnant	++++(+)	+++(+)	++(+)	++
663*	114 „ „	+++(+)	+++	++	+
652*	217 pregnant	++	+(+)	+	(+)
705	Not served nor infected	—	—	—	—
660	Pregnant, served by a clean bull	—	—	—	—

Controls	*Serum-agar dilutions*			
	1/8	1/16	1/32	1/64
Negative bovine serum	++(+)	++	(+)	Tr.
Positive „ „	++++	+++(+)	+++	++(+)
Suspension of trichomonads alone	—			
Saline-agar, with suspension ...	—			
Agar, with suspension	—			

* The titres of the infected animals were declining at the time of this test.

was possible only during the initial period of the infection and before the development of an agglutinin titre in the mucus. The presence of the agglutinin corresponded with the disappearance of the motile trichomonad. This correlation was observed in all four animals.

(*b*) *Examination for agglutinins in mucus from different parts of the vagina.* At *post-mortem* examination on heifer 710, the vagina was divided into three areas,—the cervical region, the middle portion, and the posterior part. Mucus samples were collected from each and tested for agglutinins. Table V shows that the greatest concentration of agglutinins was in the mucus collected from the cervical region.

TABLE V

SHOWING THE CONCENTRATION OF AGGLUTININS IN THE CERVICAL MUCUS

Heifer 710	*Agglutination results with various mucus-agar dilutions*			
	1/10	1/20	1/40	1/80
Cervical region ...	++++(+)	++++	++++	+++(+)
Middle portion vagina	++++	+++(+)	+++	++(+)
Posterior vagina ...	++++	+++(+)	+++	++(+)

(*c*) *Agglutination in relation to oestrous and post oestrous mucus.* A marked decrease in mucus agglutinins, sometimes to zero, as

TABLE IV

SHOWING THE REDUCTION OF THE DEMONSTRABLE AGGLUTININ CONTENT OF VAGINAL DISCHARGES COLLECTED FROM HEIFER 710, AND ITS ASSOCIATION WITH THE OCCURRENCE OF THE UTERINE TYPE OF DISCHARGE AND WITH THE RECOVERY OF MOTILE TRICHOMONADS

Days after infection	*Mucus-agar dilutions. Agglutination results*				*Recovery of organisms*	*Nature of discharge*
	1/10	1/20	1/40	1/80		
67	++(+)	++	+(+)	+	Negative D.O.*	Thick mucus uniformly cloudy
83	—	—	—	—	Positive D.O., few active organisms. Many rounded and sluggish forms on culture	Discharge more copious still mucoid, but fairly clear and moderately fluid
85	—	—	—	—	Positive D.O., many active organisms on culture	
88	—	—	—	—	Positive D.O., many organisms and all active	
89	(+)	—	—	—	Positive D.O., many organisms moderately active	
92	+++(+)	+++(+)	+++	++(+)	Negative D.O., and on culture	Thick mucus
96	Tr.	—	—	—	Positive D.O., organisms very active and exceptionally numerous	Copious clear watery discharge containing discrete flakes of pus
97	—	—	—	—		
99	++(+)	++	+(+)	(+)	Negative D.O., positive on culture showing many rounded and sluggish forms	Thick mucus uniformly cloudy
100	+++(+)	+++	++	++	Negative D.O.	

* Direct microscopical observation immediately on sampling.

detected by the mucus test, was noted in material collected at oestrus from the infected heifers. A series of these observations, made on heifer 703, is shown in Table VI.

Occasionally slight agglutination of the trichomonad suspension was detected in the post-oestrous mucus from the uninfected heifer (705) and in the infected heifers before it was possible to detect agglutinins at other periods. (Table VI). This agglutination was only observed when the mucus was heavily charged with blood. It was thought that the relatively high serum content was responsible for the non-specific agglutination as in blood agglutination test.

TABLE VI

THE NON-SPECIFIC AGGLUTINATION OF *T. foetus* BY POST-OESTROUS MUCUS HEAVILY CONTAMINATED WITH BLOOD (HEIFER 703) AND THE DISAPPEARANCE OF AGGLUTININS ASSOCIATED WITH OESTROUS MUCUS

Days after infection	*Agglutination results. Mucus-agar dilutions*				*Nature of discharge*
	1/10	1/20	1/40	1/80	
Before development of mucus agglutinins					
21	—	—	—	—	Oestrous mucus
22	++(+)	(+)	Tr.	—	Copious discharge heavily contaminated with blood
23	+	Tr.	—	—	
24	—	—	—	—	Thick mucus, no blood
After development of mucus agglutinins					
129	++++	+++(+)	+++	++(+)	Scanty vaginal mucus
131	—	—	—	—	Copious discharge of clear oestrous mucus
143	++++	+++(+)	+++	++(+)	Scanty vaginal mucus

Observations on the agglutinin extracted from vaginal mucus.

Mucus samples collected from heifer 710 after the development of an appreciable mucus titre, were resolved to give 1 : 5 mucus glucose-saline dilution and mixed with an equal volume of melted glucose-saline agar (1·5 per cent. "Davis" N. Zealand agar in glucose saline). Eight c.c. quantities of this mixture were poured into 6-inch petri dishes. The plates were allowed to solidify and glucose-saline pipetted on to the surface of each, 1·5 c.c. of saline to every 4 c.c. of the solidified mixture. The plates were then incubated for one and a half hours, left at room temperature for one and a

half hours and finally the supernatant fluid lying on the surface of each plate filtered through Whatman No. 1 filter paper. Mucus samples from an uninfected heifer were treated and extracted similarly.

Amounts of 6 c.c. of the filtered mucus extracts were heated to different temperatures for varying periods. The effect of heat on the agglutinin was assessed by the addition of 2 c.c. of a suspension of living trichomonads of the Manley strain to 2 c.c. quantities of serial dilutions of the heated extracts, which were poured into small petri dishes and incubated for one and a half hours. The petri dishes were then allowed to remain at room temperature for a further one and a half hours, when the degree of agglutination of the trichomonad suspension was assessed under the microscope as in the blood agglutination test.

No agglutination was recorded in any of the mucus extracts prepared from the uninfected heifer, and the results obtained from the infected heifer are shown in Table VII.

TABLE VII

THE EFFECT OF HEAT ON THE AGGLUTININ EXTRACTED FROM VAGINAL MUCUS OF HEIFER 710
Mucus 710 *Pooled*

Treatment	*Agglutination results with various dilutions of extracted agglutinin*					
	1/2	1/4	1/8	1/16	1/32	1/64
Untreated extract	+++(+)	+++	+++	++	(+)	Tr.
Water bath 60° C. for 15 mins.	+++(+)	+++	++(+)	+	(+)	Tr.
„ 60° C. „ 30 „	+++(+)	+++	++(+)	(+)	Tr.	Tr.
„ 100° C. „ ½ min. (Max. temp. 90° C.)	—	—	—	—	—	—
Saline suspension control ...	—	—	—	—	—	—

It will be seen that heating the extract to 60° C. for a quarter to half an hour resulted in a slight fall in titre only, while heating in a water bath at 100° C. for half a minute was sufficient to destroy the agglutinin.

The Strain Specificity of the Extracted Mucus Agglutinin.

In view of the resemblance of the Stafford to the Manley strain, it was thought that information on the possible specificity of the agglutinin might be obtained from a controlled experiment to detect difference in the agglutination of the Manley and Belfast strains by the extracted mucus agglutinin. The results which are set out in Table VIII show that there was a considerably greater agglutination of the Manley strain than the Belfast strain.

TABLE VIII

THE RELATIVE AGGLUTINATION OF THE MANLEY AND BELFAST *T. foetus* SUSPENSION BY EXTRACTED MUCUS AGGLUTININ FROM HEIFER 703

	Strain used in test suspension	*Agglutination results with various mucus extract dilutions*			
		1/10	1/20	1/40	1/80
Mucus 703 (infected heifer)	Belfast	+++(+)	++	(+)	Tr.
„ 703 „ „	Manley	++++	+++(+)	+++	+(+)
Mucus 705 (normal virgin heifer)	Belfast	—	—	—	—
Mucus 705 (normal virgin heifer)	Manley	—	—	—	—
Suspension agglutinability controls		*Agglutination results with various serum dilutions*			
		1/8	1/16	1/32	1/64
Normal rabbit serum ...	Belfast	+	(+)	Tr.	—
„ „ „ ...	Manley	+(+)	+	Tr.	—
Belfast immunised rabbit serum	Belfast	+++(+)	++(+)	++	+(+)
Belfast immunised rabbit serum	Manley	++	+(+)	+	—
Manley immunised rabbit serum	„	++++	+++	++	++
Manley immunised rabbit serum	Belfast	++	+	+	—
		Belfast	*Manley*		
Reagent controls	Agar	—	—		
	Agar-saline	—	—		
	Suspension	—	—		

DISCUSSION

The *T. foetus* infections initiated in the four virgin heifers through coitus with an infected bull have reproduced certain of the clinical features and types of disease recorded in typical field outbreaks of trichomoniasis. A characteristic example of an acute infection was seen in heifer 710, and of sub-acute infection in heifers 663 and 703. The sub-clinical infection in heifer 652 was more exceptional, but Beller, Schaaf and Scherle (1938), Beller and Schaaf (1939) and Kerr and Robertson (1943) have recorded similar results following artificial infection experiments.

The recovery of trichomonads from heifers 663 and 703 was in general agreement with the pattern of fluctuations reported by Hammond and Bartlett (1945) and Bartlett and Hammond (1945) in a group of animals with a similar history.

The presence of an agglutinin in vaginal mucus was first detected in a sample from heifer 710, but no observations had been made on this animal just after the infective service. In the other three infected animals, however, samples of mucus negative to the agglu-

tination test were collected for some time after the infective service, and observations were made on the gradual increase in titre during the course of the infection. *T. foetus* is not generally considered to penetrate the mucosa of the genital tract but to live in the surface secretions, and the accumulation of agglutinins at that site may represent a specific defensive mechanism against the organism. The agglutination which occurs when blood contaminates the mucus sample may be traceable to the serum content of the former and to be of the non-specific type observed in the blood agglutination test. The origin of the specific mucus agglutinin has still to be determined. It was demonstrated in the mucus from each infected heifer before any increase in blood agglutination titre could be detected. This suggests the development of a true local immunity but Kerr (1944) and Pierce (1946a) have shown that the skin intradermal test may also be positive without the detection of any specific blood agglutination reaction. It is also suggested by Kerr and Robertson (1943, 1946), that the antibody to *T. foetus* in the circulating blood may become fixed in the dermis. The presence of a high level of non-specific agglutinin and the use of an agglutination test lacking in acute sensitivity renders it reasonable to suppose that specific serum agglutinins may circulate and still remain undetected. In a similar way agglutinins might also become fixed in the vaginal mucosa and eventually appear in the secretions originating there, although the immunity was actually of a humoral origin. A correlation appears to exist between the level of mucus agglutinin, and the *eventual* development of specific blood agglutinins, for heifer 710, which developed the greatest concentration of mucus agglutinins, eventually became "above normal" to the blood agglutination test. Heifer 703 also showed strong mucus agglutinin reactions and became positive to the agglutination test; heifers 652 and 663, on the other hand, developed weaker mucus agglutinins and showed no specific blood agglutinins.

The association of the disappearance of mucus agglutinins in vaginal discharges with the change from vaginal mucus to the more watery uterine type was of considerable interest in the explanation of many phenomena seen in experimental observations and in the field. The recovery of organisms at times when mucus was of more fluid consistency has been observed by several authors, (Andrews and Miller, 1938; Bartlett and Hammond, 1945; Hammond and Bartlett, 1945; Madsen and Jensen, 1940; Pierce, 1946a) and has been shown by these experiments to coincide with the disappearance or marked reduction of agglutinins, while the rapidity with which the vaginal population of the trichomonads was reduced at the cessation of the uterine discharge may be correlated with the disappearance of this diluting factor and the return of the vaginal mucus rich in agglutinins. This offers a reasonable explanation for the frequent finding of organisms in the so-called typical trichomonad discharge which is of a watery consistency

often with flakes of pus, and for the absence of trichomonads in other types of discharge.

Any resistance to reinfection that might be acquired as the result of the development of the mucus agglutinin has yet to be determined.

The cultural observations carried out on the discharges from the animals in these experiments were incomplete since they were only made occasionally. It was noted, however, that discharges which were negative on direct examination and positive after culture were most frequent in the intial stages of the development of the mucus agglutinin when the titre was still low. The cultured organisms when first observed were usually round or oval in outline and showed very slight motility. It was considered, therefore, that the agglutinin at certain concentrations might influenece motility and even morphology of the organism, making it very difficult to detect by direct examination ; this would explain the accepted view that discharges should be examined as quickly as possible after sampling, and would account for the variable duration of the motility of the organism at room temperature in different mucus samples.

SUMMARY

An agglutinin to *T. foetus* has been demonstrated in the vaginal discharges from four heifers infected with trichomoniasis by natural service. It was not demonstrable in two uninfected heifers by the same technique. The appearance of the agglutinin coincided with the disappearance of the active organism, and was associated with the type of discharge that is thought to be of vaginal rather than uterine origin.

A sudden disappearance or considerable reduction in concentration of agglutinin in the mucus was associated either with the copious discharges that accompany a return to oestrus, or with the appearance of the uterine type of discharge, the latter frequently being associated with the reappearance of active organisms in large numbers. On the cessation of the uterine discharge the agglutinins reappeared and trichomonads could no longer be demonstrated.

Diffusion products from a 1/10 dilution of mucus in agar contain agglutinins capable of causing echelon agglutination and immobilisation of a trichomonad suspension after two and a half hours exposure, or to cause marked agglutination with reduction of motility.

Some correlation has been established between the degree of development of the mucus agglutinin and the serological response of the infected heifers. Immunologically the mucus and serum agglutins developed have been related to the strain of *T. foetus* isolated. The extracted agglutinin was found to be relatively resistant to heating at 60° C. but rapidly destroyed by heating to 100° C. Cultural methods of demonstrating the motile organism were some-

times superior to direct observation, usually after the development of the agglutinin in the vaginal discharges.

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THE EXAMINATION OF STAPHYLOCOCCI OF ANIMAL ORIGIN WITH PARTICULAR REGARD TO THE DETERMINATION OF CRITERIA OF PATHOGENICITY

By

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INTRODUCTION

Owing to their ubiquitous nature and the difficulty experienced in their classification, much work has been devoted to the study of staphylococci obtained from human sources in order to distinguish pathogenic from non-pathogenic strains. Various properties, such as pigment production, fermentation of lactose and mannitol, liquefaction of gelatin, haemolysis on blood agar, production of filtrable toxins and their recognition by haemolytic, skin-necrotising and lethal effects have all been critically examined. Other properties such as the demonstration of leucocidin, fibrinolysin, and the lethal effect of the staphylococci themselves on experimental animals have also been studied.

Coagulation of plasma by staphylococci first observed by Loeb (1903–04), and later noted by Much (1908) to be a property of pathogenic strains only, has now assumed much importance. Recent work on the classification of staphylococci particularly that by Chapman *et al.* (1934), Cruikshank (1937), Cowan (1938), Fairbrother (1940) and Christie and Keogh (1940) has shown that while some of the other tests are of considerable value, the only absolute criterion of pathogenicity is the production of coagulase. Some workers now accept the division of the genus Staphylococcus, as suggested by Fairbrother (1940), into *Staphylococcus pyogenes* (coagulase positive strains) and *Staphylococcus saprophyticus* (coagulase negative strains).

A search of the literature reveals that very little of the work done on staphylococci of animal origin has been directed to discovering criteria of pathogenicity. Dumas (1914) examined the cultural and biochemical reactions of 17 strains, eight of which were of animal and nine of human origin; the toxins produced by these same strains were studied by Nicolle and Cesari (1914). Minett (1936) examined 51 pathogenic strains from various animal species with particular reference to their toxins but did not determine their capacity to form coagulase. He concluded that the production of β toxin was a characteristic feature of haemolytic staphylococci obtained from animals and that strains from the dog could be distinguished by their greater proteolytic power. Minett (1937) later examined 41 non-pathogenic, and an unspecified number of pathogenic strains isolated from the cow's udder. The coagulase tube test was included in the examination, but no detailed account was given as to the results obtained. Cruikshank (1937) examined six animal strains of unspecified origin for coagulase production by the tube test; all were coagulase-positive. Cowan (1938), while classifying human strains, examined 20 of diverse animal origin for pigment production, mannitol fermentation, gelatin liquefaction, Voges-Proskauer reaction, haemolysin, coagulase (tube test) and precipitins.

* Wellcome Research Fellow Veterinary Educational Trust.

Field and Smith (1945) examined 124 strains of staphylococci isolated from the cow's udder by the coagulase tube test and by the rapid slide test, using bovine plasma, and also 110 strains from various domestic animals and man by the slide test only but using a variety of plasmas. They concluded that coagulase production was confined to pathogenic staphylococci and confirmed the findings of Birch-Hirschfield (1934), Berger (1943) and Cadness-Graves *et al.* (1943) working with human strains of staphylococci, as to the value of the rapid slide test. Recently, Christie *et al.* (1946) have reported the examination of 1,027 strains of staphylococci from human and animal sources, all of which were tested for coagulase production (tube test), haemolysis on sheep blood agar, formation of pigment and fibrinolysin and fermentation of mannitol. A number of them were also examined for haemotoxin production and pathogenicity to mice. These authors concluded that a strain may produce coagulase and still be non-pathogenic and that all pathogenic strains produce haemolysis on sheep blood agar while non-pathogenic ones do not.

ORIGIN OF MATERIAL

One hundred and seventy-three strains, classified provisionally as 110 pathogenic and 63 non-pathogenic, were examined. The former were isolated in pure and plentiful culture from active infections or lesions where little doubt existed that they were responsible for the pathological condition; not more than one strain from any one outbreak of infection was included in the group. The 63 strains provisionally regarded as non-pathogenic were selected by means of the coagulase tube test using human plasma and were picked from coagulase negative colonies appearing on blood agar plates streaked with swabs taken from normal healthy animals. The origin of the strains is given in Table I.

TABLE I

ORIGIN OF STRAINS

Nature of Strain	*Dog*	*Cattle*	*Sheep*	*Poultry*	*Goat*	*Horse*	*Total*
Pathogenic	24	41	23	13	7	2	110
Non-pathogenic ...	15	24	12	7	4	1	63
Total	39	65	35	20	11	3	173

METHODS

The staphylococci were examined for pigment production, growth at 17° C, fermentation reactions, indole production, gelatin liquefaction, action on litmus milk, growth on MacConkey's agar, liquefaction of solid serum, coagulase production, fibrinolysin, haemolysis on blood agar, haemotoxin, dermotoxin, lethal toxin, and for the lethal effect of the organisms in the manner described below.

Pigment Production. Strains were streaked on nutrient agar in Petri plates which were incubated for 24 hours at 37·5° C. and then left at room temperature for eight days.

Growth at 17° C. Nutrient agar slopes seeded with one 2 mm. loopful of an 18 hour broth culture of each of the strains were incubated at 17° C. for 30 days, and observed daily. For the purpose of recording results, a culture was considered to have "grown" when it was equivalent in amount to that obtained by incubating a "typical" staphylococcus culture at 37·5° C. for 18 hours.

Liquefaction of Solid Serum. Dog, horse, ox and sheep sera were used for this test so that the action of all the strains on each of these sera could be compared. Incubation was at 37·5° C. for 14 days.

Fermentation Tests. The substances used were arabinose, xylose, glucose, galactose, saccharose, maltose, lactose, raffinose, inulin, mannitol, dulcitol, sorbitol, salicin and inositol.

Incubation was carried out at 37·5° C. for fermentation tests, gelatin liquefaction and growth in litmus milk. Observations were made over a period of 21 days.

Coagulose Production.

Tube Test. The procedure used was Gillespie's (1943) modification of the method devised by Fisk (1940). Tests were put up in duplicate using bovine and human plasmas and positive and negative controls were always kept. Plasma from sterile blood containing 0·4 per cent. sodium citrate was used within 24 hours of collection in order to obviate false positive reactions which old plasma may give (Christie and Keogh, 1940).

Rapid Slide Test. A similar method to that adopted by Berger (1943) was employed. Where the nature of the reaction appeared at all doubtful, tests were repeated using fibrinogen solution and serum instead of plasma. This was to distinguish clumping of organisms due to coagulation of fibrinogen from true agglutination, the latter being observed with serum but not with fibrinogen, and the former with fibrinogen but not with serum. Tests were carried out as in the tube test, using bovine and human plasmas.

Plate Test. The original test as devised by Penfold (1944) was used, about 20 strains being tested on the same plate.

Fibrinolysin.

Tube Test. The method of Tillett and Garner (1933) was employed using sheep, instead of human, plasma. Examination for evidence of dissolution of the clot was carried out over a period of 48 hours but not beyond owing to the tendency for sterile fibrin clots to autolyse after this time.

Plate Test. The original method of Christie and Wilson (1941) was used.

Haemolysis on Blood Agar. Broth cultures of the strains to be tested were spot inoculated on to 10 per cent. sheep blood agar plates, about ten strains being tested on each. The plates were examined after 24 hours' incubation at 37·5° C. and then left at room temperature for a further period of four days.

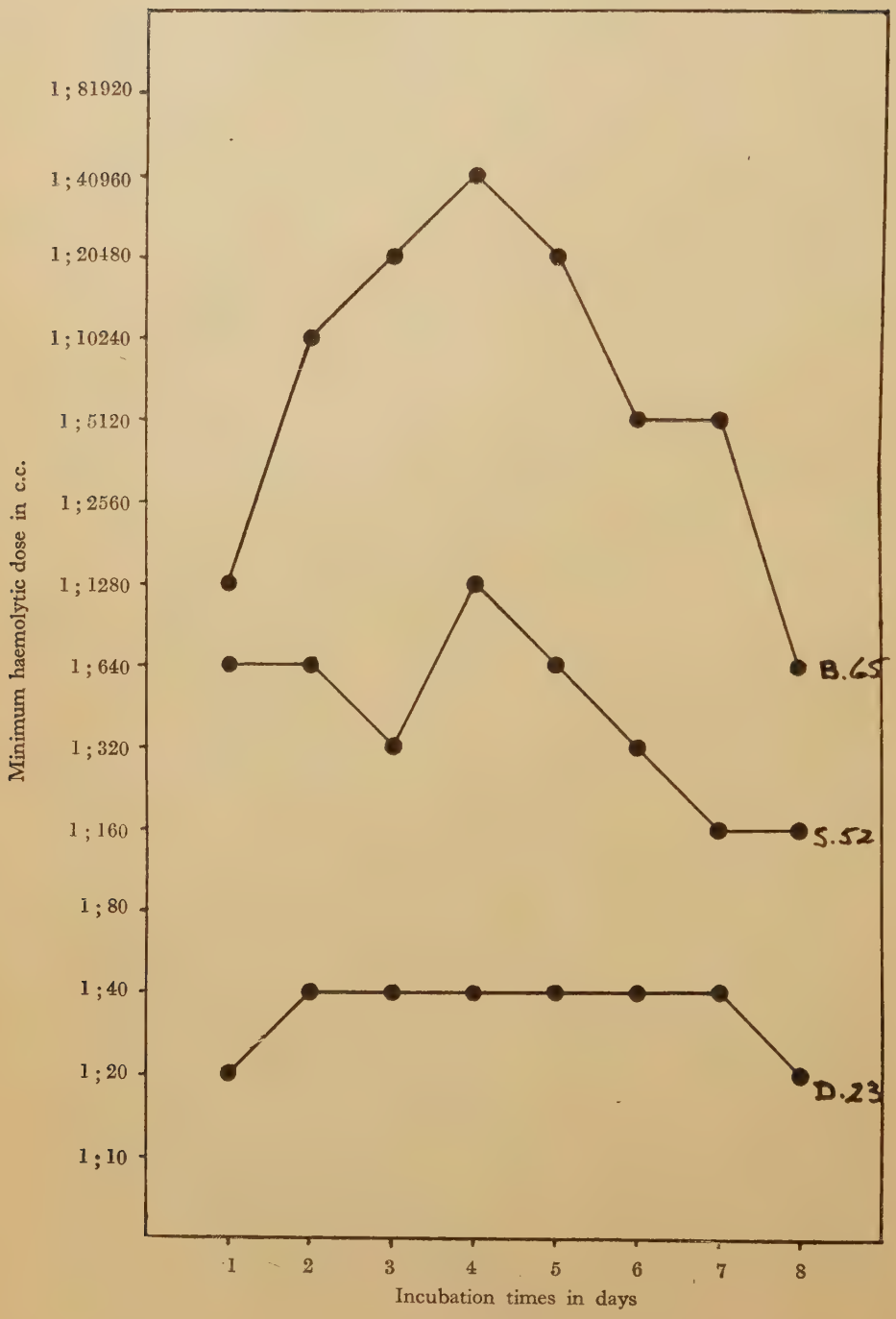
Preparation of Toxin. The method used was similar to that

described by Burnet (1931), but the medium in 20 c.c. amounts was contained in 8 oz. sloped medicine flats. Toxin was extracted by adding 2 c.c. of normal saline, leaving for half an hour and expressing the juice which was centrifugalised at 3,500 revolutions per minute for one hour. The supernatant fluid was then removed and stored in the ice chest. Various times have been suggested as the optimum for harvesting toxin by this method. Burnet allowed 24 hours but some workers prefer longer periods of incubation. Consequently, it was decided to carry out an initial experiment using three strains, B65, D23 and S62 in order to ascertain by haemotoxin titrations their optimum times of toxin production. Each strain was seeded into eight bottles of soft agar which were incubated for periods of from one to eight days during which the production of haemotoxin was determined by titrations at daily intervals carried out as described below. Account was taken of α toxin only, since Bryce and Rountree (1936) noted and the author has also observed that the amount of β toxin produced by a given strain of staphylococcus varies considerably even under similar conditions. The results expressed in Graph I indicate that four days was the most satisfactory incubation period for the three strains tested. Consequently this was the time adopted when examining other strains for toxin production.

Haemotoxin Examination. Two series of agglutination tubes were set up in duplicate to give dilutions in normal saline of from 1 : 5 to 1 : 20,480 of the centrifugate to be tested. An equal volume of a 1 per cent. suspension of washed rabbit erythrocytes in sterile normal saline was added to one set of tubes and of sheep erythrocytes to the other set, the final volume in each tube being 1 c.c. Readings were taken after incubating the tubes for two hours at 37·5° C. in a water bath and again after leaving them at room temperature overnight. The end point was taken as that dilution in which 50 per cent. of the erythrocytes had been haemolysed, although this was not always sharply defined. Centrifugates were considered to contain only β toxin when the hot/cold phenomenon of Bigger *et al.* (1927) was observed in the tubes containing sheep erythrocytes but no haemolysis occurred with rabbit erythrocytes. Those which haemolysed rabbit erythrocytes and also showed the hot/cold phenomenon with sheep erythrocytes were noted as containing both α and β toxins while those which lysed rabbit erythrocytes and also sheep erythrocytes to a lesser degree and did not show the hot/cold phenomenon when left overnight, were recorded as producers of α toxin only. Where doubt existed as to the presence of β toxin, a series of dilutions were set up in the presence of an excess of α antitoxin. The tubes were incubated in a water bath at 37·5° C. for half an hour when the sheep erythrocytes were added after which they were incubated for a further two hours, left overnight and then read. Any haemolysis of the sheep cells was taken to indicate the presence of β toxin.

GRAPH I

Effect of Incubation Time on Toxin Production



B.65 = Cattle strain. S.52 = Sheep strain. D.23 = Dog strain.

Dermotoxin. Using a dental type syringe 0·1 c.c. of undiluted centrifugate was injected intradermally into a white depilated guinea pig, 12 samples being tested simultaneously on the same animal. Observations were made over a period of seven days. A typical positive reaction appeared as an area of necrosis surrounded by an hyperaemic zone, a negative one as no obvious change at the site of injection. A third type of reaction was recorded, namely, the appearance of an indurated area which persisted for several days.

Lethal Toxin. This was detected by injecting mice and rabbits intravenously, a fixed dose 0·4 c.c. of centrifugate being used for mice and various amounts for rabbits. Observations were carried out for a period of ten days.

Lethal Effect of Organisms. A suspension of a fresh 18-hour agar slope culture in sterile normal saline solution with a density equivalent to that of Brown's tube No. 5 was used in doses of 0·3 c.c. intraperitoneally for mice and 1·0 c.c. intravenously for rabbits. Observations were made over a period of two months after injection.

RESULTS

Pigment Production. Of 110 pathogenic strains 79 (71·8 per cent.) were golden, 29 (26·4 per cent.) white and two (1·8 per cent.) yellow. Only three out of 24 dog strains were golden, and in their case the pigment became perceptible only after three days' incubation whereas in the other 76 strains it was easily observed at 24 hours.

Of the 63 non-pathogenic strains, 17 (27 per cent.) were golden, 44 (69·9 per cent.) white and two (3·1 per cent.) yellow. Golden pigment was obvious in all the 17 strains at 24 hours including the dog ones. It was noted that five of the dog non-pathogenic strains produced golden pigment, while only three of the pathogenic ones did so.

Growth at 17° C. The non-pathogenic strains grew much more rapidly than the pathogenic ones at 17° C., 47 (74·6 per cent.) of the former having grown in 48 hours, but none of the latter. By the 5th day all the non-pathogenic strains had grown, but only 36 (32.7 per cent.) of the pathogenic ones leaving 18 (16·3 per cent.) which grew between the 5th and 10th day, 46 (41·8 per cent.) between the 10th and 30th days and ten which produced no visible growth.

Fermentation Reactions. All the 173 strains examined produced acid but no gas in glucose, galactose, saccharose, maltose and lactose, while a variable number of the pathogenic and non-pathogenic strains split all the other fermentable substances which were used for test except arabinose which was attacked by 12 of the strains classified as non-pathogenic, but by none of the pathogenic strains. The results indicate briefly that the fermentation reactions do not serve to classify the pathogenic strains, or to distinguish them from the non-pathogenic ones. All the poultry pathogenic strains, however, fermented salicin rapidly but only a small proportion of the other

strains attacked it and then usually after a longer period of incubation. Although 105 out of 110 pathogenic strains produced acid in mannitol, 54 out of 63 non-pathogenic ones also did so. All the positive reactions with inulin were slight.

TABLE II

SUMMARY OF FERMENTATION REACTIONS

Substance	*Pathogenic strains*		*Non-pathogenic strains*	
	+	—	+	—
Arabinose ...	0	110	12	51
Xylose	7	103	12	51
Glucose	110	0	63	0
Galactose ...	110	0	63	0
Saccharose ...	110	0	63	0
Maltose	110	0	63	0
Lactose	110	0	63	0
Raffinose ...	22	88	23	40
Inulin	40	70	17	46
Mannitol ...	105	5	54	9
Dulcitol	13	97	4	59
Sorbitol	47	63	6	57
Salicin	34	76	14	49
Inositol	31	79	6	57
Trehalose * ...	14	4	4	2

+ = Acid but no gas production.
* Only 24 strains were tested with trehalose.

Indole Production. All the 173 strains examined gave a negative reaction to this test.

Liquefaction of Gelatin. Of the 110 pathogenic strains which were examined all but four liquefied gelatin. Thirty-five (55·6 per cent.) out of 63 non-pathogenic strains also liquefied it and 28 did not. Dog pathogenic strains appeared to act most rapidly and had all completed the process within four days. Some of the other pathogenic strains were much slower two of them requiring as long as 20 days.

Litmus Milk. Of 110 pathogenic strains, 109 produced an acid clot in litmus milk followed by contraction of the clot to a much smaller volume, leaving a clear supernatent whey (A.C. + R.). Further incubation resulted in a gradual decolorisation of the litmus. One pathogenic poultry strain gave an acid reaction without any clotting of the milk. Forty-seven (74·6 per cent.) of the 63 non-pathogenic strains gave the A.C. + R. reaction on litmus milk, while 16 (25·4 per cent.) produced acid only.

MacConkey's Medium. All the strains produced a good growth of lactose-fermenting colonies after 18 hours' incubation at 37·5° C.

Liquefaction of Solid Serum

None of the 63 strains classified as non-pathogenic was able to liquefy solid serum. Twenty-four (21·9 per cent.) of the patho-

TABLE III
THE LIQUEFACTION OF SOLID SERUM

Pathogenic strains	*Positive*	*Negative*
Dog	14	10
Cattle	2	39
Sheep	0	23
Poultry	8	5
Goat	0	7
Horse	0	2
Total	24 (21·9 per cent.)	86 (78·1 per cent.)

genic strains did so, while 86 (78·1 per cent.) did not. None of the strains isolated from sheep, horse, or goat, and only two of the 41 cattle strains, were positive. The two latter were slow in producing their effect, only a slight depression appearing in the medium by the third day of incubation and becoming a little more obvious by the 14th. Fourteen out of 24 dog strains and eight out of 13 from poultry liquefied solid serum rapidly and copiously causing deep depressions in the medium within 24 hours of incubation, which increased considerably with further incubation. The results shown in Table III were obtained with either dog, horse, or ox serum but dog serum was the most readily liquefied followed by horse and then ox. Sheep serum was acted upon much more slowly, three days' incubation being required before the positive dog and poultry strains had produced any visible change. Similar results were obtained with different batches of sheep serum.

Fibrinolysin.

Tube Test. None of the 173 strains caused any obvious lysis of the fibrin clots within 48 hours' incubation. A number of the tests were repeated using a larger volume of broth culture but results were still negative.

Plate Test. Sixty-one (55·5 per cent.) of the pathogenic strains produced fibrinolysin when tested by this method and 49 (44·5 per cent.) did not. All the non-pathogenic strains were negative. All the dog, poultry, goat and horse pathogenic strains but only eight out of 41 of those from cattle and seven out of 23 from sheep were able to lyse fibrin. Positive strains produced a zone of complete clearing around the colony varying in thickness usually from 1 to 3 mm. after 24 hours and increasing only slightly with further incubation. All the strains which liquefied solid serum also lysed fibrin when tested by this method. The only two cattle strains involved produced exceptionally wide zones of fibrinolysis.

Coagulase Production.

Tube Test. All the 110 pathogenic strains were coagulase positive and as explained above, all the 63 strains classified as non-pathogenic

were by definition negative to this test. All the coagulase positive strains produced a firm solid coagulum with human plasma and all but 18 a similar reaction with bovine plasma. These 18 produced large flake coagulation, the flakes being deposited at the bottom of the tube with a clear uncoagulated supernatent fluid above. They gave similar results when retested with several different samples of bovine plasma and fresh broth cultures, and also when the plasma was used in a dilution of 1 : 5 instead of 1 : 10. This type of reaction occurred within three hours incubation except for three strains with which it appeared overnight.

TABLE IV

THE EFFECT OF TIME ON THE COAGULASE TUBE TEST

Pathogenic strains	*Human plasma*		*Bovine plasma*	
	Positive within three hours	*Positive only after leaving overnight*	*Positive within three hours*	*Positive only after leaving overnight*
Dog	0	24	20	4
Cattle	41	0	33	8
Sheep	23	0	20	3
Poultry	9	4	13	0
Goat	7	0	6	1
Horse	2	0	2	0
Total	82 (74·5 per cent.)	28 (25·5 per cent.)	94 (85·5 per cent.)	16 (14·5 per cent.)

Table IV shows that 94 (85·5 per cent.) of the pathogenic strains coagulated ox plasma within three hours incubation while the remaining 16 (14·5 per cent.) did so only after standing at room temperature overnight. Eight-two (74.5 per cent.) coagulated human plasma within three hours and 28 (25·5 per cent.) overnight. The pathogenic dog strains coagulated human plasma only after leaving overnight while 20 out of the 24 coagulated bovine plasma within three hours. The tests of the dog strains on human plasma were repeated, using a different batch of plasma and fresh broth cultures, with similar results. Despite the care taken in performing the tube tests, when the 63 strains classed as non-pathogenic were tested for the first time, three false positive reactions with bovine plasma and two with human plasma were noted. These appeared only after leaving at room temperature overnight. Several retests were made with consistently negative results.

Rapid Slide Test. The 107 pathogenic strains tested were all coagulase positive and the 63 non-pathogenic strains all negative. Three pathogenic strains yielded only granular suspensions in normal saline even after being repeatedly subcultured and thus were not suitable for this test ; the addition of plasma, however,

caused the immediate production of a strong coagulase-positive type of reaction. With five exceptions all the pathogenic strains reacted strongly immediately the plasma and suspension were mixed and were easily recognised. The exceptions gave weak, delayed reactions in the form of small clumps resembling a weak agglutination effect. As the same thing occurred with fibrinogen solution but not with serum, it was evidently a true coagulase reaction and not agglutination.

All the non-pathogenic strains maintained a perfectly homogeneous suspension when plasma was added. Bovine and human plasmas gave completely identical results when used for the rapid slide test.

Plate Test. All pathogenic strains were positive and all non-pathogenic ones negative to this test. Positive reactions varied somewhat with different strains; some produced dense, opaque, sharply delimited zones of varying width around the colony; others much fainter and usually wider zones which gradually merged into the surrounding medium. In most cases the width beyond the edge of the colony after 24 hours' incubation was from 1·5 to 4 mm. and had rarely increased by more than 1 mm. at 48 hours. Three strains coagulated fibrinogen subcolonially only with bovine plasma and produced a very thin band of opacity around the colony with human plasma. Three others were negative at 24 hours with human plasma but showed wide, very faint zones at 48 hours. In contrast, two strains negative at 24 hours with human plasma had dense but narrow zones at 48 hours. The 18 strains which gave large flake coagulation with bovine plasma in the tube test gave poor reactions with it in the plate test; in the case of fourteen of them they were perceptible as wide but faint zones only. As in the tube test, however, these strains gave strong positive reactions with human plasma after 48 hours.

Haemolysis on Sheep Blood Agar. For purposes of classification *only,* a clear zone of haemolysis is referred to as α type, a semi-clear zone as β type and a clear zone surrounded by a semi-clear zone as $\alpha\beta$ type. Seventy-five (68·2 per cent.) of the pathogenic strains showed $\alpha\beta$ type haemolysis on sheep blood agar after 24 hours' incubation while 26 (23·6 per cent.) produced β type only. Two pathogenic strains, both from goats, produced α type only. Of the pathogenic strains from poultry, seven were non-haemolytic and the remaining six of the $\alpha\beta$ type. The zones of α type were usually 0·5 to 2 mm. wide beyond the edge of the colony at 24 hours and the β zones were from 3 to 5 mm. wide. On further incubation both zones, particularly β type, tended to widen. Some of the strains showing β type haemolysis only at 24 hours developed a very slight zone of apparent α type at 48 hours, but, owing to the uncertainty of interpretation this was not recorded.

Ten (16·9 per cent.) of the non-pathogenic strains produced haemolysis of the α type, with zones usually 0·5 to 2 mm. wide at 24

TABLE V

HAEMOLYTIC EFFECT ON SHEEP BLOOD AGAR

Origin of strains	*Pathogenic* (110)				*Non-pathogenic* (63)			
	α type only	*αβ type*	*β type only*	*Negative*	*α type only*	*αβ type*	*β type only*	*Negative*
Dog	0	23	1	0	3	0	0	12
Cattle ...	0	23	18	0	6	0	0	18
Sheep ...	0	16	7	0	0	0	0	12
Poultry ...	0	6	0	7	0	0	0	7
Goat... ...	2	5	0	0	1	0	0	3
Horse ...	0	2	0	0	0	0	0	1
Total ...	2 (1·8 per cent.)	75 (68·2 per cent.)	26 (23·6 per cent.)	7 (6·4 per cent.)	10 (16·9 per cent.)	0	0	53 (83·1 per cent.)

hours. The remainder of the strains classed as non-pathogenic were non-haemolytic.

A number of pathogenic strains were seeded on to blood agar plates made with ox, guinea pig, rabbit, horse and chicken blood for comparison with sheep blood. Ox and chicken blood appeared similar to sheep blood, rabbit blood was totally resistant to the β type but more sensitive to the α type than sheep blood, while the strains had no haemolytic effect on guinea pig blood. No β type haemolysis was noted with horse blood and the zones of α type were narrower than with sheep blood.

Haemotoxin Production. Of the 110 pathogenic strains examined 91 (82·7 per cent.) formed both α and β toxins, ten (9·1 per cent.) β toxin only and two (1·8 per cent.), both from goats, α toxin only. Seven (6·4 per cent.) strains, all from poultry, did not yield centrifugates capable of lysing either sheep or rabbit erythrocytes. The ten strains which produced no detectable α toxin but only β toxin were all from dogs. The remainder of the dog pathogenic strains produced only a small amount of α toxin, no centrifugate having a higher titre than 1 : 40 for a 1 per cent. rabbit erythrocyte suspension. All the cattle, sheep, horse and goat strains, and the haemolytic ones from poultry yielded considerable amounts of α toxin, titres ranging from 1 : 160 to 1 : 40,960. Whilst the amount of α toxin in successive batches from the same strain was very uniform, the yield of β toxin was found to vary considerably, thus confirming the observations of Bryce and Rountree (1936) on this latter toxin.

None of the strains classed as non-pathogenic yielded centrifugates lytic for sheep or rabbit erythrocytes. Table VI shows that there is complete correlation between β type haemolysis on sheep blood agar and the production of β toxin as judged by titration tests. However, the presence or absence of a clear zone of haemolysis

("α type") on sheep blood agar is no reliable indication of the capacity of a strain to produce α toxin; 23 dog pathogenic strains produced α as well as β type haemolysis on sheep blood agar, yet ten of them were later proved to produce β toxin only. Also, 18 cattle and seven sheep strains which produced no obvious clear zone of haemolysis on sheep blood agar, were found to be powerful producers of α toxin. None of the 63 non-pathogenic strains produced α toxin, yet ten of them gave a clear type of haemolysis on sheep blood agar.

TABLE VI

A COMPARISON OF THE EFFECT OF THE PATHOGENIC STAPHYLOCOCCI ON SHEEP BLOOD AGAR AND THEIR HAEMOTOXIN PRODUCTION

Strains	*10 per cent. sheep blood agar*				*Haemotoxin tests*			
	α type only	*α + β type*	*β type only*	*Negative*	*α toxin only*	*α + β toxin*	*β toxin only*	*Negative*
Dog ...	0	23	1	0	0	14	10	0
Cattle ...	0	23	18	0	0	41	0	0
Sheep ...	0	16	7	0	0	23	0	0
Poultry ...	0	6	0	7	0	6	0	7
Goat ...	2	5	0	0	2	5	0	0
Horse ...	0	2	0	0	0	2	0	0
Total ...	2	75	26	7	2	91	10	7

Dermatoxin Production. Centrifugates from 56 (50·9 per cent.) of the pathogenic strains gave a typical positive necrotic reaction; 23 (20·9 per cent.) produced a hyperaemic zone which later became indurated and 31 (28·2 per cent.) were negative. The non-haemolytic pathogenic poultry strains were negative as were all the pathogenic dog strains.

The 31 negative strains were also tested on rabbits with similar results. None of the non-pathogenic strains produced demonstrable dermotoxin.

The centrifugates, prepared from two cattle strains, B65 and B37, were titrated in order to discover the minimum necrotising dose (M.N.D.) and its relationship to the minimum haemolytic dose (M.H.D.) for 1 per cent. rabbit corpuscles. For B65 the M.H.D. was 1:40,960 c.c. and the M.N.D. 1:640 c.c.; for B37 the corresponding figures were 1:5,120 c.c. and 1:80 c.c. Thus in each case the M.N.D. was 64 times the M.H.D. This relationship held good with all the other centrifugates examined in that only those with an α titre of 1:640 or more produced typical necrosis when injected intradermally into guinea pigs in 0·1 c.c. amounts. Those with α titres between 1:160 and 1:640 produced indurated areas only at the site of injection and below this level there was no visible effect.

TABLE VII

DERMOTOXIN TESTS (PATHOGENIC STRAINS)

Strains	*Positive*	*Indurated*	*Negative*
Dog	0	0	24
Cattle	29	12	0
Sheep	16	7	0
Poultry—			
Positive haemolysin	4	2	0
Negative haemolysin	0	0	7
Goat	5	2	0
Horse	2	0	0
Total	56 (50·9 per cent.)	23 (20·9 per cent.)	31 (28·2 per cent.)

Lethal Effect of Toxin on Mice. Centrifugates from 82 pathogenic strains were tested in a dose of 0·4 c.c. intravenously and thirty-four (41·2 per cent.) proved fatal. Those from the pathogenic dog strains and the non-haemolytic pathogenic poultry strains were without effect, but from the remaining 51 strains, 34 were lethal.

TABLE VIII

LETHAL EFFECT OF TOXIN ON MICE (PATHOGENIC STRAINS)

Strains	*Number tested**	*Died*	*Survived*
Dog	24	0	24
Cattle	20	14	6
Sheep	18	12	6
Poultry—			
Positive haemotoxin	6	3	3
Negative haemotoxin	7	0	7
Goat	5	3	2
Horse	2	2	0
Total	82	34 (41·2 per cent.)	48 (58·8 per cent.)

* All strains were not tested.

Although observations were continued for ten days, no deaths were noted later than eight hours after injection, the majority occurring within two hours and several within two to five minutes. The minimum lethal dose (M.L.D.) for mice of the centrifugates from the strains B65 and B37 was found to be approximately 512 times the M.H.D. and in conformity with this result the 34 centrifugates which were lethal to mice in a dose of 0·4 c.c. intravenously haemolysed rabbit erythrocytes in dilutions of 1 : 1,280 or more. An inverse relationship was noted between the α toxin content of a centrifugate and the survival time of mice. The β toxin content, however, appeared to play no part in the lethal effect. Centrifugates from 23 strains classed as non-pathogenic were all non-lethal to mice,

Lethal Effect of Toxin on Rabbits. Owing to shortage of rabbits the only centrifugates tested were from eight pathogenic strains (five dog and three poultry) which were non-lethal to mice. All failed to kill rabbits in a dose of 4 c.c. Eight non-pathogenic strains, tested in a similar manner, also proved non-lethal.

Lethal Effect of Organisms on Mice. Sixty (66·6 per cent.) out of 90 pathogenic strains were lethal. The 24 pathogenic dog strains and six out of seven non-haemolytic pathogenic poultry strains were non-lethal, but all strains from other species which were tested as well as four haemolytic poultry strains proved fatal (Table IX). The non-lethal strains were also tested by intravenous injection with negative results. The lethal strains killed within 48 hours excepting two which took five days. Cultures were made from the heart's blood of all mice which died and a pure growth of staphylococci was obtained from each.

The 63 non-pathogenic strains had no effect on mice.

TABLE IX

LETHAL EFFECT OF THE PATHOGENIC STRAINS ON MICE

Strains	*Number tested*	*Lethal*	*Non-lethal*
Dog	24	0	24
Cattle	30	30	0
Sheep	19	19	0
Poultry—			
+ haemotoxin ...	4	4	0
— haemotoxin ...	7	1	6
Goat	4	4	0
Horse	2	2	0
Total	90	60 (66·6 per cent.)	30 (33·3 per cent.)

Lethal Effect of Organisms on Rabbits. Five pathogenic strains from sheep and two from cattle were fatal to rabbits within 18 hours of intravenous injection whereas six from the dog and two non-haemolytic ones from poultry were without effect as were eight non-pathogenic strains.

DISCUSSION

The results of all the tests taken as a whole show that the production of coagulase is the only absolute criterion of pathogenicity among strains of staphylococci ; all the 110 pathogenic strains were coagulase positive while the remaining 63 which were provisionally accepted in the first instance as non-pathogenic on the results of the coagulase tube test, were subsequently shown to lack many of the properties possessed by the majority of the pathogenic strains. Complete correlation existed between the results obtained by the slide, tube and plate tests for coagulase and each of these tests had its advantages. The great value of the slide test is rapidity and

ease of performance ; suspected colonies may be tested at primary isolation and a result obtained in a few moments. All but five of the pathogenic strains gave unmistakable positive reactions. The five weak reactors were not tested until some months after isolation so it is conjecturable as to whether or not their powers of coagulase production may by then have declined. These five strains, however, gave strong positive reactions to the tube test which tends to confirm that it is more sensitive than the slide test. This fact has been noted by several earlier workers, particularly Cadness-Graves *et al.* (1943) who consequently recommend that all strains which are negative to the slide test be subjected to the tube test. Birch-Hirschfield (1934) and Berger (1943), however, found complete correlation between the results of the two tests, but both these authors examined a smaller number of strains than did Cadness-Graves *et al.* A disadvantage of the slide test is that occasional strains are met with which will not form an homgeneous suspension in normal saline, and are therefore unsuitable for tests ; three such strains were encountered among the 173 examined. Compared with the rapid slide test, the tube test demands much more care and preparation. The necessity of making a final observation after leaving the tubes at room temperature overnight is shown by the fact that positive reactions were not given by 28 (25·5 per cent.) strains with human plasma and 16 (14·5 per cent.) with bovine plasma before this time. This longer period of observation may also result very occasionally in a false positive reaction ; three strains produced this effect with bovine plasma and two with human plasma when first tested, but gave completely negative results at several subsequent tests. It may be considered advisable to retest all late positive reactors to the tube test, particularly if they are negative to the slide test. The fact that 18 strains persistently produced large flake coagulation with different batches of bovine plasma is difficult to understand. Evidently they were not poor producers of coagulase since they reacted strongly with human plasma, and were also positive to the possibly less sensitive slide test ; their reaction with bovine plasma by the plate test was, however, poor, usually becoming noticeable only after 48 hours' incubation although here again with human plasma it was strong. Neither can their behaviour be attributed to the bovine plasma used since other strains tested with it at the same time gave a firm coagulum. The plate test has the disadvantage of sometimes requiring 48 hours incubation before results can be read and positive reactions are occasionally difficult to discern. The writer's results are somewhat similar to those obtained with human strains by Penfold (1944) who found that 94 per cent. of tube-positive staphylococci produced easily recognised rings of opacity while the remainder, along with some of the coagulase negative strains, produced sub-colonial coagulation. None of the coagulase negative strains noted in this present work produced a sub-colonial coagulation.

The results show clearly that fermentation tests, pigment production, and action on gelatin and litmus milk do not serve to differentiate pathogenic from non-pathogenic staphylococci. This is in agreement with the more recent work on this subject. The liquefaction of solid serum and fibrin, the production of β type haemolysis on sheep blood agar and of haemotoxin, dermotoxin and lethal toxin were, however, properties confined to the pathogenic strains and then shared only by some of them.

The production of clear "α type" haemolysis on sheep blood agar was not restricted to pathogenic strains; nor did its presence indicate capacity to produce α toxin as judged by the effect of centrifugates on rabbit erythrocytes. Moreover, seven pathogenic strains were encountered which were non-haemolytic. It is, therefore, evident that neither the presence of haemolysis on blood agar, other than the darkened β type nor the absence of haemolysis on this medium is of value in the classification of animal staphylococci and the expression "haemolytic staphlycoccus" might well be dropped as it is of no significance. The results of the toxin tests confirm the close relationship between α toxin, dermotoxin and lethal toxin which has been noted by many workers.

With the exception of the dog strains and the non-haemolytic pathogenic poultry strains, the lethal effect of the staphylococci on mice follows completely the initial classification of the strains into pathogenic and non-pathogenic. There can be little doubt that these dog and poultry strains were pathogenic to the animals from which they were isolated and, in fact, several of the poultry strains had been obtained from the heart blood of birds killed in the acute stage of disease and examined immediately. Minett (1936), also, did not find mouse virulence to be an absolute test for pathogenicity. Christie *et al.* (1946), however, placed great reliance on this test which may possibly account for their observation that some non-pathogenic strains produce coagulase, a finding which is not borne out by the very large amount of work published by others on this subject. The results also illustrate the close relationship between α toxin production and the lethal effect of the organisms on mice: all the dog strains which were poor producers of α toxin and the non-haemolytic poultry strains, with one exception, were without effect on mice while all the other pathogenic strains which were good producers of α toxin were lethal.

The pathogenic dog strains formed a fairly distinctive group in that they nearly all produced white pigment, formed much β toxin but little or no α toxin, no demonstrable dermotoxin or lethal toxin, were non-fatal to mice and rabbits, were mainly active liquefiers of solid serum and all produced fibrinolysin. The pathogenic poultry strains as a group also had several distinctive features; the majority were active liquefiers of solid serum, all fermented salicin rapidly, and over half of them were non-haemolytic and did not form any demonstrable toxins.

SUMMARY

One hundred and seventy-three strains of staphylococci of animal origin were provisionally classified as 110 pathogenic and 63 non-pathogenic strains. The former were designated as such because they were all isolated in pure and plentiful culture from active lesions or infections while the latter were selected from coagulase-negative colonies appearing on blood agar plates streaked with swabs taken from normal animals.

The results, taken as a whole, bear out the initial classification and illustrate the value of the coagulase test, applied in either the tube, rapid slide, or plate method, as the only single reliable test for distinguishing pathogenic from non-pathogenic strains. The appearance of β type haemolysis on sheep blood agar, liquefaction of solid serum, production of fibrinolysin, haemotoxin, dermotoxin and lethal toxin and the lethal effect of the organisms themselves were properties exhibited only by pathogenic strains and then only by a proportion of them. Consequently the possession of any one of these properties may be accepted as evidence of a pathogenic staphylococcus although its absence is without significance. Pigment production, action on litmus milk, the liquefaction of gelatin, the fermentation of mannitol and other substances were of little value in the detection of pathogenic strains. The non-pathogenic, as a general rule, grew much more rapidly at 17° C. than the pathogenic ones.

The pathogenic strains from the dog and those from poultry showed certain characteristics which tended to distinguish them from cattle, sheep, goat and horse pathogenic strains. The four latter groups were indistinguishable from each other.

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OBSERVATIONS ON THE AETIOLOGY OF LYMPHOMATOSIS*

I.—STUDY ON "CHICK DISEASE"

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INTRODUCTION

Blakemore (1939, 1945) has established that many fowls affected with lymphomatosis harbour a virus which when injected into young chicks induces acute focal lesions of the liver and heart. Evidence was submitted in support of the theory that these lesions represented an early acute reaction to a virus responsible for lymphomatosis in older fowls. Blakemore's work served to focus attention upon this disease which is here referred to as "chick disease."

The study of lymphomatosis has been handicapped by the difficulties in reproducing the typical disease, the long incubation period, the problems of maintaining adequate isolation and the difficulty in obtaining experimental stock free of lymphomatosis.

The "chick disease" lends itself more readily to experimental study in that the virus can be readily demonstrated in chicks, the incubation period is short and the disease can be constantly transferred by inoculation or pen contact. Much information has been obtained on its nature and on methods of control. If the theory of the relationship of "chick disease" to lymphomatosis could be substantiated, this information could be utilised in the control of lymphomatosis. It must be admitted, however, that experimental data indicates that lymphomatosis may occur in the absence of "chick disease" virus. Until the question is finally settled it is obviously undesirable to apply the knowledge gained from a study of "chick disease" to the control of lymphomatosis.

The symptoms, lesions and method of demonstrating "chick disease" virus have been described by Blakemore (1939, 1941, 1945) and Glover (1940) and do not warrant further attention here. The present paper deals with the persistence of the virus in the tissues of inoculated chickens, methods of spread, the resistance of the virus to certain physical and chemical conditions and the effect of certain sulphonamides in controlling the infection.

VIRULENCE AND PERSISTENCE OF VIRUS STRAINS IN CHICKENS

Most of the strains of virus studied by Blakemore were enhanced in virulence and caused a high mortality by passage through chickens of his P. strain, but when injected into chickens of other strains

* The term lymphomatosis is here used to include those manifestations of the avian leukosis complex grouped under the sub-heading lymphomatosis by Jungherr, Durant and Lee, 1941.

the mortality was lower. A similar virus recovered from a turkey affected with neurolymphomatosis (Glover, 1940) did not change qualitatively during 16 passages through Brown Leghorn and Plymouth Rock chicks, since only 14 out of 198 inoculated chickens died.

From 1940 to 1946, 63 " chick disease " virus strains have been examined by the writer ; 54 were obtained from fowls affected with lymphomatosis, four from adult fowls showing no evidence of lymphomatosis and five from young chickens which exhibited lesions of " chick disease." These figures do not indicate the relative frequency of the virus in lymphomatous and non-lymphomatous fowls since the majority of the fowls examined for the virus were affected with lymphomatosis and chicks were not examined unless they showed macroscopic evidence of the disease. All 63 strains when injected into chicks induced lesions similar to those described by Blakemore, although some minor differences such as the severity of the lesions and the degree of involvement of the spleen were noted. . No marked change in virulence occurred as a result of serial passage of 62 of the strains through Rhode Island Red chicks. In the case of one strain, however, a marked rise in virulence took place so that the virus produced a fatal infection in most chicks.

The disease ran a typical course in inoculated chickens. Visceral lesions usually appeared within three to four days of infection ; they increased in number and extent and were most conspicuous during the second week. Between the 15th and 30th days the lesions regressed and were frequently inconspicuous or absent by the 30th day. During one period 1,136 chicks (seven to ten days old) were inoculated with various virus strains, but only 103 (9·1 per cent.) died during the subsequent 14 days. It is evident that mortality due to the virus was low since this figure includes chicks which would normally have been expected to die, in addition to those which died as a result of the mechanical interference. The " chick disease " virus provoked marked visceral lesions in chickens of six weeks or younger, whereas in older chickens the lesions were much less conspicuous and could readily be overlooked. The virus produced no gross visceral lesions when injected into normal adult fowls.

Although visceral lesions disappeared within a month of infection, most chickens continued to harbour the virus for a much longer period. This fact was commented upon by Blakemore (1945), who noted that the most satisfactory way of maintaining the virus was to inject it into two to three month old chickens from which it could be recovered several months later. An experiment was carried out in which young chicks were inoculated with " chick disease " virus. The faeces were examined for the virus periodically, and the liver, heart and spleen when the fowls died or were killed. No difficulty was experienced in identifying the virus by the inoculation of chicks with faeces suspensions of some fowls. The virus was also demon-

strated in the tissues of three out of ten fowls examined 400 to 664 days after inoculation. Whilst there is no doubt that the virus can be safely maintained in growing chickens it is obvious that strict isolation of the host fowl is essential if this method is adopted.

The virus could not be demonstrated in the tissues of ducklings, rabbits, guinea-pigs or mice 14 days after injection.

TABLE I

TRANSMISSION OF THE "CHICK DISEASE" VIRUS THROUGH THE EGGS OF INOCULATED HENS

Period when eggs layed	*Number of eggs incubated*	*Infertile*	*Dead embryos*	*Chicks hatched*	*Results*
During 14 days before inoculation of hens	60	3	14	43	All killed 5th-20th day after hatching. No gross lesions of "chick disease." Chicks inoculation expt. Negative
1st - 14th day after inoculation	52	6	13	33	Deaths commenced on 8th day. All dead by 37th day. Most chicks showed lesions of "chick disease." Chick inoculation expt. Positive
15th - 28th day after inoculation	52	1	20	31	Deaths commenced on 2nd day. All dead by 42nd day. Most showed lesions of "chick disease." Chick inoculation expt. Positive
56th - 72nd day after inoculation	29	2	13	14	Three died. Remainder killed 10th-40th day. Some showed lesions of "chick disease." Chick inoculation expt. Positive
80th - 96th day after inoculation	28	6	12	10	Two died. Remainder killed 10th-38th day. No gross lesions of "chick disease." Chick inoculation expt. Negative
180th-194th day after inoculation	38	3	10	25	Two died. Remainder killed 10th-40th day. No gross lesions of "chick disease." Chick inoculation expt. Negative

NATURAL AND ARTIFICIAL TRANSMISSION OF THE "CHICK DISEASE"

Experiments have confirmed Blakemore's observations that "chick disease" can be successfully transmitted by any parenteral route; in addition, the disease caused by the virulent strain readily spreads to healthy chicks by pen contact. Transmission of virus strains of lower pathogenicity by this method was uncertain.

In view of the fact that inoculated chickens continue to harbour the virus for long periods observations were made on the possibility that the virus might be transmitted through the medium of the hatching egg. Nine R.I.R. hens were injected intraperitoneally with 1·0 ml. of a liver suspension of a chick infected with a virulent strain of "chick disease" virus. Hatching eggs were collected during the 14 days before and 194 days following inoculation. The virus produced no obvious change in the hens. The eggs were incubated in a disinfected incubator, and when hatched the chicks were removed to clean isolation brooders. When 10 to 40 days old all surviving chicks were killed and examined for lesions of "chick disease." Specimens of liver and heart removed from each chick, pooled, triturated with sand and suspended in broth were injected into healthy chicks in order to detect the virus. The results given in Table I indicate that transmission through the egg took place. It cannot be assumed that a high proportion of the chicks were infected before hatching since conditions were favourable for contact transmission immediately after hatching. In a similar experiment hatching eggs were obtained from three farm flocks which were known to harbour the virus. The eggs were incubated in disinfected incubators and the chicks examined for the virus in the same way. The virus was detected in pooled liver suspension of chicks hatched from 100 eggs obtained from one source, but negative results were obtained in the other groups.

The possibility of the infection being transmitted on the egg shell was also studied. The shells of nine-day embryonated eggs were painted with undiluted embryonic fluid of chick embryo which was infective at a dilution of 2×10^{-7} per ml. Incubation was continued at 103·5° F. and two eggs were withdrawn 24, 48, and 72 hours later. The shells were carefully removed, ground with sand and suspended in broth. The supernatant fluid of all samples when injected into young chicks failed to induce visceral lesions. From this experiment and the evidence that the virus has only limited ability to survive outside the chicken, it seems probable that the virus was carried in the egg contents rather than on the shell.

Since "chick disease" spreads readily by pen contact, an attempt was made to demonstrate the virus in the faeces of infected chicks. Fresh faeces, collected 24 to 336 hours after intraperitoneal inoculation of infective tissue suspension, were extracted with broth and the coarse particles allowed to separate by gravity. The supernatant fluid was injected intraperitoneally into healthy susceptible chicks,

In order to avoid severe mortality from peritonitis it was necessary to use chicks of 20 to 30 days old. All chicks were killed and examined for lesions ten days after injection. The results with faeces freshly collected, after storage at 33 to 35° F. and at brooder temperature (85° F.) are shown in Table II. The data indicates that the virus is present at about the same time that gross visceral lesions become evident, *i.e.*, 48 hours after inoculation and that the virus activity is rapidly lost in stored faeces. Further experiments demonstrated that some inoculated chickens continued to excrete the virus in their faeces for many months.

TABLE II

THE PRESENCE OF "CHICK DISEASE" VIRUS IN THE FAECES OF INFECTED CHICKS

Time after inoculation of donor chicks that faeces were collected	*Virus in fresh faeces*	*Virus in faeces stored at 33°-35° F. for*			*Virus in faeces stored at 80-85° F. for*		
		48 *hours*	96 *hours*	144 *hours*	8 *hours*	12 *hours*	24 *hours*
24 hours	—						
48 „	+						
96 „	+	+	—	—	—	—	—
144 „	+	+	—	—	—	—	—
216 „	+						
336 „	+						

THE INFECTIVE AGENT

All attempts to cultivate a micro-organism from chicks infected with "chick disease" have failed and no inclusion bodies have been demonstrated in the tissues. Some of the characters of the infective agent have been recorded by Blakemore (1945). Moreover, virus can be cultivated in embryonated hen's eggs (McGaughey, 1941). All the strains which have been studied by the writer have failed to induce lesions on the chorio-allantoic membranes or to increase embryonic mortality significantly, but visceral lesions similar to those seen in growing chicks developed in the embryo. The extra-embryonic fluids were found to be a rich source of virus and provided convenient suspensions for filtration, serum neutralisation and other experiments.

The virus in undiluted embryonic fluids would pass Gradocol membranes of 0·4μ A.P.D. Membranes of smaller pore diameters consistently gave negative results. The virus was inactivated by heat in a water bath at 49° C. for 15 minutes but was only incompletely inactivated at 47° C. for 60 minutes. The virus in embryonic fluids was inactivated within one hour by potassium permanganate 0·01 per cent., hydrogen peroxide (10 vol.) 5 per cent., formalin (40 per cent.) 0·1 per cent. In two experiments conducted by Glover (1940) the virus in infected chick liver withstood desiccation,

Five attempts were made to preserve infective embryonic fluid and chick liver by desiccation from the frozen state but in all attempts the virus failed to survive the drying process. The resistance of the virus in chick liver under varying conditions was as follows: at 2° to 4° C., infective seven days later but not at 10 days; at 2° to 0° C., infective 18 days later but not at 38 days; frozen in normal horse serum and stored at —2° C., invariably infective eight weeks later (one specimen remained infective after storage in this manner for 101 days).

Liver tissue preserved in 50 per cent. glycerol saline and stored at 1° to 2° C. was infective after 12 days but not after 17 days.

THE CURATIVE EFFECT OF SULPHONAMIDES ON CHICKS INFECTED WITH "CHICK DISEASE" VIRUS

Asplin (1944) reported that the disease in chicks could be effectively treated with certain sulphonamides. Sixty-three strains of virus were adapted to chicks and have been used in different experiments. As they produced lesions regularly within a few days the effect of chemotherapeutic substances on the development of the disease could be readily assessed.

All the chicks used in these experiments were 6 to 12 days old R.I.R. × L.S. cockerels or unsexed R.I.R. chicks which were maintained in separate units, precautions being taken to avoid contact infection. They were infected by the intraperitoneal route with 0·5 ml. of a 1 per cent. suspension of affected liver. In most instances they were fed on a standard mash containing the drug but in certain experiments the sodium derivative of the sulphonamide was dissolved in the drinking water. Treatment commenced immediately after infection and was continued for seven to ten days when all surviving chicks were killed and the disease diagnosed upon the presence of gross lesions.

The results of experiments which were carried out using a variety of sulphonamides are summarised in Table III. Strain 8 virus was used in Experiments 1 to 12 and the sulphonamides prevented the development of lesions in the following concentrations per oz. of food: Sulphadiazine 0·01 g., sulphamezathine 0·01 g., sulphathiazole 0·06 g., sulphapyridine and sulphaguanidine 0·25 g. Sulphanilamide had no effect on "chick disease" at 0·25 g. per oz. of food, and furthermore it was toxic when fed at this rate. The observation of the effect of sulphadiazine on "chick disease" has been repeated using 63 strains of virus from other sources. One strain (T. 70) was found to be much less susceptible than the remainder (Table III, Experiment 13) since it was sensitive to sulphadiazine only when the drug was present in the food at the rate of 0·25 g. per oz. The results were confirmed in experiments in which drinking water was replaced by solutions of the sodium derivatives of the sulphonamides (Table IV).

Several attempts were made to demonstrate the virus in groups

TABLE III

PREVENTION OF "CHICK DISEASE" WITH SULPHONAMIDES

Experimental drug	*Grammes per oz. of food*													*Control untreated*
	1/4	1/8	1/16	1/25	1/32	1/48	1/64	1/75	1/106	1/128	1/150	1/200	1/250	
1. Sulphanilamide	3/4	3/5	4/5		5/5									4/5
2. Sulphaguanidine	0/5	4/5	4/5		5/5		5/5							5/5
3. Sulphapyridine	0/5	5/5	3/5		4/5	3/4								4/5
4. Sulphathiazole	1/5	0/5	0/5		1/5									5/5
5. Sulphathiazole (chicks six days old)		0/5	0/5		4/6		4/6			5/6				4/4
6. Sulphathiazole				1/5		2/5			4/5			4/5		5/5
7. Sulphamezathine	0/5	0/5	0/5		0/5	1/4								5/5
8. „				0/5		0/5		0/5	1/5					5/5
9. „						0/5			2/5		4/5	4/5		5/7
10. Sulphadiazine	0/5	0·5	0/5		0/5	0/5								5/5
11. „				0/4		0/5		0/5	0/5		3/5			2/5
12. „									0/5		2/5	2/5	3/5	4/5
13. „	0/4	3/5	3/5		4/5									4/5

Expts. 1-12 were carried out with Strain 8 virus; another strain (T.70) was used for Expt. 13

The figures indicate the number of chicks showing macroscopic lesions of "chick disease" and the number in each group

TABLE IV

PREVENTION OF THE "CHICK DISEASE" WITH SOLUTIONS OF CERTAIN SULPHONAMIDES REPLACING DRINKING WATER

Sulphonamide (Sodium salt)	*Concentration mgs. per 100 ml.*							*Untreated control*
	500	250	100	50	25	10	5	
Sulphapyridine	0/5	1/5	3/5					4/5
Sulphathiazole	0/5	0/5	0/5	4/5	3/5			4/5
Sulphamezathine				0/5	0/5	0/5	1/5	4/5
Sulphadiazine			0/5	0/5	0/5	0/5	1/4	4/5

The figures represent the number of chicks showing gross lesions of "chick disease" and the total number in each group.

of chicks which had been dosed with sulphadiazine for the seven days immediately following inoculation, but all with negative results. When dosing was delayed until four days after infection, treatment for seven days resulted in the arrest and resolution of visceral lesions but in one experiment three chickens in a group of 34 were still harbouring the virus. Chicks which had recovered following treatment with sulphadiazine were fully susceptible to re-infection with the same virus strain.

The bacteriostatic action of sulphonamides upon streptococci is inhibited both *in vitro* and *in vivo* by *p.* aminobenzoic acid (PAB). The curative effect of sulphamezathine and sulphapyrazine on coccidiosis was also inhibited by this substance (Horton Smith and Boyland, 1946). An experiment was carried out to test the inhibiting effect of *p.* aminobenzoic acid upon sulphonamides in the treatment of "chick disease." The results (Table V) show that the

TABLE V

THE INHIBITORY EFFECT OF *p.* AMINOBENZOIC ACID ON SULPHONAMIDES IN THE TREATMENT OF THE "CHICK DISEASE"

p. Aminobenzoic acid in food at level of	*Sulphonamides in food*		
	Sulphathiazole 1/8 *gm./oz.*	*Sulphadiazine* 1/8 *gm./oz.*	*Sulphamezathine* 1/8 *gm./oz.*
1/16 gm./oz.		5/5	
1/32 „		5/5	4/5
1/48 „		5/5	5/5
1/64 „		2/6	3/5
1/96 „		3/5	3/5
1/144 „	4/5	1/5	2/5
1/200 „	3/5		
1/240 „	5/5	0/5	0/5
1/400 „	5/5	0/5	0/5
1/800 „	0/5		
1/1000 „	0/5		
1/2000 „	0/5		
No *p.* aminobenzoic acid	0/5	0/5	0/5
Untreated controls	4/5	5/5	4/5

curative effect of sulphamezathine, sulphadiazine and sulphathiazole was neutralised by PAB. The amount necessary to inhibit the effect of sulphadiazine and sulphamezathine at a dose of 1/8 g. per oz. of food was approximately four times more than that necessary to neutralise the effect of the same dose of sulphathiazole. This is in keeping with the observation (Table III) that the dose of sulphathiazole necessary to prevent the development of lesions of the "chick disease" is four to five times greater than that of sulphadiazine or sulphamezathine. Virus suspended in a solution of sulphadiazine (100 mg. per 100 ml.) remained infective after four days' storage at 2° to 4° C.).

Penicillin had no curative effect in chicks infected with this virus.

DISCUSSION

Observations on the association of "chick disease" virus with lymphomatosis and the characters of the agent are in agreement with the findings of Blakemore. The disease induced in chicks by 62 of the 63 virus strains studied was characterised by low mortality, the only marked symptom being a check in growth rate. The transmission of the virus by the carrier hen to the chick via the egg and the ready spread by pen contact provides means for widespread dissemination of the disease. It appears probable, however, that while "chick disease" virus may be widely distributed in poultry flocks, it is only exceptionally the cause of chick losses.

Following serial passage one virus strain became greatly enhanced in virulence and produced a rapidly fatal infection in chicks. It is conceivable that under field conditions "chick disease" virus may produce serious disease in young chicks. Sulphonamides should prove of value in checking such outbreaks. The drugs might be given to the breeding stock or to the young chicks. As the data reported in the paper indicate that the virus is readily destroyed by heat and disinfectants and only survives for short periods in faeces or fowl tissues, control of the disease in chicks should not prove difficult.

Experiments are reported elsewhere in which sulphadiazine dosing completely failed to influence the incidence of lymphomatosis in experimentally inoculated chickens. Recommendations for the control of lymphomatosis based upon knowledge gained with "chick disease" virus cannot be justified at the present stage.

SUMMARY

Observations on 63 strains of "chick disease" virus confirm the findings of Blakemore with regard to the nature of the agent and the disease in young chicks. The mortality in chicks infected with 62 of the strains was lower than that recorded by Blakemore. Chickens infected with one virus strain continued to harbour it for as long as 664 days.

The virus could be readily transferred by parenteral inoculation or pen contact, and transmission from hen to chick via the hatching egg was demonstrated. Observations were made upon the survival of the virus under various conditions.

The " chick disease " could be successfully treated with certain sulphonamides. The curative effect of the sulphonamides was inhibited by *p*. aminobenzoic acid.

It is pointed out that in view of the doubt regarding the relationship of " chick disease " virus to typical lymphomatosis, information gained from its study cannot be applied to the control of lymphomatosis.

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OBSERVATIONS ON THE AETIOLOGY OF LYMPHOMATOSIS

II.—THE ASSOCIATION OF "CHICK DISEASE" VIRUS WITH FIELD CASES OF LYMPHOMATOSIS

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INTRODUCTION

The presence of a virus capable of stimulating an acute inflammatory reaction in young chicks has been demonstrated in the tissues of fowls affected with lymphomatosis (Blakemore, 1939, 1941, 1945; Asplin, 1944, 1945). Blakemore (1945) recorded this virus in 12 out of 14 outbreaks of lymphomatosis in East Anglian flocks. Asplin (1945) reported the demonstration of a similar agent from four out of 20 lymphomatous fowls obtained from flocks in Michigan and Massachusetts, U.S.A.

The studies reported in this paper represent an attempt to assess the relative frequency of "chick disease" virus in lymphomatous and non-lymphomatous fowls, obtained from flocks in different areas of Britain. Observations were also made upon the clinical effect of dosing lymphomatous fowls with sulphadiazine and upon the incidence of "chick disease" virus in sulphadiazine-treated fowls.

EXPERIMENTAL

In 1943, cases of lymphomatosis, obtained from widely separated areas of Britain, were divided into two groups. The first group was composed of fowls which were killed and examined immediately after arrival at the laboratory. The second was dosed with 0·5 g. sulphadiazine per day by mouth for ten days; they were killed and examined 24 hours after the last dose. A third group consisted of healthy fowls, or fowls affected with conditions other than lymphomatosis, also killed and examined immediately on arrival. They consisted mainly of fowls submitted as suspected cases of lymphomatosis, or culls from flocks in which cases had been diagnosed, but in which no lesions of this disease were detected. This group also included healthy fowls from flocks believed to be free from lymphomatosis.

Since diagnosis was based upon the demonstration of gross or microscopic lesions of lymphomatosis after death, the fowls were not finally classified into their respective groups until the pathological examination was completed.

METHOD OF EXAMINATION

Clinical. A record was kept of the severity of symptoms shown by fowls on arrival at the laboratory and after treatment with sulphadiazine.

Pathological. All reactors to *S. pullorum* antigen or tuberculin were immediately discarded. After death the fowls were subjected to a detailed macroscopic examination, specimens of nerve, liver, heart muscle and tumour (if present) were taken for microscopic examination. Cultures were made to eliminate the common bacterial infections of poultry.

Chick Inoculation Tests. The virus producing the acute inflammatory reaction in chicks referred to earlier can readily be adapted to young chicks. In most instances the mortality amongst infected chicks is negligible but if they are killed within a few days of infection characterstic gross lesions can be observed in the liver and heart.

In these experiments specimens of liver, heart muscle, nerve and tumour (if present) were taken immediately after death of the donor fowl. They were pooled, triturated with sand and suspended in sterile broth : 0·5 ml. of the suspension was injected intraperitoneally into each of five or six young chicks. All chicks were killed seven to ten days later and examined for evidence of " chick disease." If no gross lesions were observed in chicks of the first passage group further serial transmission was carried out. The donor fowl was recorded as negative for the chick virus only after three negative serial transmissions in chicks.

When a strain of " chick disease " virus had been identified infective tissues were injected into two groups of at least five chicks each. One group was dosed with sulphadiazine, given in the food at a level of 0·5 per cent. of the diet ; the other group served as an untreated control. This dose of sulphadiazine represented approximately ten times that which had been found to protect chicks against strains of " chick disease " virus previously studied.

SERUM NEUTRALISATION TESTS

Neutralising antibodies to " chick disease " virus can be demonstrated in the sera of some fowls (Asplin, 1947). The " chick disease " strain for neutralisation experiments was obtained from a chicken affected with visceral lymphomatosis. It had been transferred in serial passage more than 250 times and constantly reproduced lesions of the chick disease. It could be cultivated in embryonated eggs. The embryonic fluid of infected embryos was found to be a convenient source of virus for serum neutralisation experiments.

METHOD AND MATERIALS

The inoculum for infecting embryonated eggs was a suspension of infected chick liver (one part) in saline (100 parts). Ten day embryonated hen eggs were inoculated with 0·1 ml. introduced into the extra embryonic fluids through a hole drilled in the shell above the air sac. The hole was sealed with paraffin wax and the

eggs incubated at 103° F. for a further 48 hours. Before harvesting fluid, eggs were candled and all with dead germs were discarded. The shell over the air sac was removed and the embryonic fluid pipetted off, care being taken to avoid rupture of the blood vesesls. The fluid from six to ten eggs was pooled and lightly centrifuged. The supernatant fluid was removed and employed as the virus suspension in neutralisation experiments.

The tests sera were obtained from blood withdrawn by heart puncture from fowls immediately before *post-mortem* examination. They were inactivated at 55° C. for 30 minutes. The virus suspension was diluted in tenfold serial dilutions with sterile saline. Equal volumes of diluted virus suspension and undiluted test serum were mixed to make final virus dilutions over the range 10^{-2} to 10^{-7} and were incubated at 37° C. for one hour. Each serum-virus mixture was inoculated into a group of five seven to ten day old chicks, receiving 0·5 ml. by intraperitoneal injection. A control series received virus diluted over the same range with sterile saline only. All chicks were killed seven to ten days later and examined for lesions of "chick disease." The end point of the titration was taken to be the greatest dilution of virus 0·5 ml. of which would produce lesions in one or more of the five test chicks. The results are shown in Table 4.

RESULTS

Paralysed fowls when housed and fed in individual cages generally showed a temporary improvement. Only a few lymphomatous fowls were treated with sulphadiazine and maintained under observation for several weeks and in these the symptoms became more marked.

Difficulty was experienced in obtaining a supply of suitable paralysed fowls. Those which were obtained were frequently isolated cases from different sources and exhibited symptoms varying widely in severity. In fowls such as these dosing with sulphadiazine for ten days produced no marked change.

The data presented in Table I show that the chick inoculation test demonstrated " chick disease " virus in the tissues of 26 out of 67 (38·8 per cent.) of lymphomatous fowls which had received no treatment with sulphadiazine. Fowls yielding strains of " chick disease " virus were obtained from 23 different sources in widely separated districts ranging from Cornwall to East Anglia, Sussex to Forfarshire. Tissues of 22 lymphomatous fowls examined immediately after ten days dosing with sulphadiazine were all negative to the chick inoculation test. Forty-one undosed fowls in which no evidence of lymphomatosis was detected were examined by the same method. These fowls had been obtained from 34 different flocks, 14 of which were apparently healthy while 27 were affected with conditions other than lymphomatosis. The " chick disease " virus was demonstrated in two (4·9 per cent.) of these fowls. It is clear from

TABLE I

THE INCIDENCE OF THE "CHICK DISEASE" VIRUS IN LYMPHOMATOUS AND NON-LYMPHOMATOUS FOWLS DETERMINED BY INJECTION OF SUSPENSIONS OF DONOR'S TISSUES INTO YOUNG CHICKS

Donors	*No. of Donors*	*Groups of recipient chicks developing lesions of the "Chick Disease"*		*Groups of recipient chicks showing no evidence of "Chick Disease"*	
		No.	*Per cent.*	*No.*	*Per cent.*
Lymphomatous. Not dosed	67	26	38·8	41	61·2
Lymphomatous dosed. Sulphadiazine, 10 daily doses of 0·5 g.	22	0	0	22	100
Non-lymphomatous. Not dosed	41	2	4·9	39	95·1

TABLE II

DATA ON THE PRESENCE OF THE "CHICK DISEASE" VIRUS IN UNDOSED LYMPHOMATOUS FOWLS

Donor		*Virus identified from farm*	*Lesions of "C D." in recipient chicks*	*Neutralisation test. No. of chick infective doses of virus neutralised*
County of origin	*Lesions*			
Bedford	V.O.	—	—	10,000
Bedford	O	—	—	10,000
Bucks	N	—	—	100
Bucks	N	+	+	>1,000
Cornwall ...	N	+	+	>10,000
Dorset	N	—	—	0
Essex	N.V.	+	+	10,000
Essex	V	—	—	0
Essex	V	—	—	0
Essex	N	—	—	0
Forfar	N	+	+	10,000
Forfar	V.O.	+	—	1,000
Gloucester ...	N.V.	—	—	0
Leicester ...	N	—	—	0
Middlesex ...	N.V.	—	—	10
Notts	N	+	+	1,000
Oxford	V	+	+	1,000
Somerset ...	N.V.O.	+	+	1,000
Somerset ...	V.O.	+	+	100
Somerset ...	N	—	—	0
Suffolk	N	—	—	10
Surrey	V.O.	—	—	10
Surrey	V	—	—	0
Surrey	V	—	—	100
Surrey	V	—	—	100
Sussex	N	+	+	>1,000
Sussex	N	+	+	>100
Sussex	N	+	+	>100
Yorkshire ...	O	—	—	0

N = Neural lymphomatosis.
V = Visceral lymphomatosis.
O = Ocular lymphomatosis.

TABLE III

DATA ON THE INCIDENCE OF THE "CHICK DISEASE" VIRUS IN SULPHADIAZINE DOSED LYMPHOMATOUS FOWLS

Donor		*Virus identified from farm*	*Lesions of "C.D." in recipient chicks*	*Neutralisation tests. No. of chicks infective doses neutralised*
County of origin	*Lesions*			
Berkshire ...	N	—	—	0
Cornwall ...	O	+	—	10,000
Essex	N	—	—	>1,000
Forfar	N	+	—	10,000
Hertford ...	N	—	—	0
Somerset ...	N	—	—	0
Somerset ...	Osteo-petrosis	—	—	0
Somerset ...	N.V.	—	—	10,000
Worcester ...	N.V.	+	—	>100,000

N = Neural lymphomatosis.
V = Visceral lymphomatosis.
O = Ocular lymphomatosis.

TABLE IV

DATA ON THE INCIDENCE OF THE "CHICK DISEASE" VIRUS IN UNDOSED NON-LYMPHOMATOUS FOWLS

Donor		*Virus identified from farm*	*Lesions of "C.D." in recipient chicks*	*Neutralisation tests. No. of chicks infective doses neutralised*
County of origin	*Lesions*			
Cumberland	Intestinal parasitism	+	+	100
Dorset ...	Vitamin A deficiency	—	—	0
Dorset ...	No lesions	—	—	0
Dorset ...	No lesions	—	—	10
Dorset ...	No lesions	—	—	0
Essex ...	No lesions	—	—	0
Essex ...	Intestinal parasitism	—	—	100
Herts ...	No lesions	—	—	1,000
Kent ...	No lesions	—	—	0
Notts ...	Intestinal parasitism	+	—	100
Notts ...	Intestinal parasitism	+	—	10
Somerset ...	Vitamin A deficiency	—	—	100,000
Surrey ...	No lesions	—	—	0
Surrey ...	Skeletal deformity	—	—	10
Surrey ...	Injury	—	—	1,000
Surrey ...	Erythroleukosis	—	—	0
Surrey ...	No lesions	—	—	100
Surrey ...	No lesions	—	—	10
Surrey ...	No lesions	—	—	0
Surrey ...	No lesions	—	—	0
Sussex ...	No lesions	—	—	10

these experiments that "chick disease" virus can more readily be demonstrated in the tissues of lymphomatous fowls than in non lymphomatous fowls.

The sera of 60 fowls were also examined for neutralising antibody. The data (Tables 2, 3, 4 and 5 show) that all fowls in which "chick disease" virus was demonstrated by chick inoculation, also showed evidence of neutralising antibody. In addition, the sera of

TABLE V

SUMMARY OF THE DATA CONTAINED IN TABLES II, III AND IV, UPON THE INCIDENCE OF THE "CHICK DISEASE" VIRUS IN LYMPHOMATOUS AND NON-LYMPHOMATOUS FOWLS

Test for "Chick Disease" virus		*Donor fowls*		
Chick inoculation	*Serum neutralisation**	*Lymphomatous not dosed*	*Lymphomatous dosed sulphadiazine*	*Non-lymphomatous*
+	+	11	0	1
—	+	6	5	6
—	—	13	4	14
+	—	0	0	0

* Neutralisation of 100 or more chick infective doses considered positive.

six out of 30 lymphomatous fowls which were negative to the chick inoculation test possessed a neutralising property. The results also show that 13 out of 30 lymphomatous fowls were negative to both tests for this virus. Nine sulphadiazine dosed lymphomatous fowls examined by both tests were negative to the chick inoculation test: five showed evidence of serum neutralising antibody and four were negative. Altogether 39 lymphomatous fowls were examined by both methods; 22 (56·4 per cent.) were positive to one or both tests for "chick disease" virus. Of 21 non-lymphomatous fowls only one was positive to both tests, six were positive to the serum neutralisation test and negative to the chick inoculation and 14 (66·6 per cent.) were negative to both tests. In considering these results it should be recalled that many of the non-lymphomatous fowls had been received from farms on which lymphomatosis had previously been diagnosed. A point of possibly greater significance is that 17 (43·6 per cent.) of the lymphomatous fowls examined by both tests showed no evidence of "chick disease" virus.

The 28 fowls from which "chick disease" vrius was demonstrated by direct inoculation were obtained from 26 different sources (*i.e.*, 26 strains). The disease induced in chicks by all 26 virus strains was effectively treated with sulphadiazine, although one of these strains (T.70) was found to be more resistant to sulphadiazine than the remainder.

DISCUSSION

The results of the present investigation together with the findings of Blakemore (1945) indicate a wide distribution of "chick disease" virus particularly in fowls affected with lymphomatosis. No survey of the incidence of "chick disease" virus in young chicks has been made but it has been identified in a few cases by Blakemore (1945) and by the writer. In one flock the disease affected chicks of each successive hatch causing a mortality of 10 to 20 per cent. at the age of two to four weeks; in another the virus was identified in chicks suffering from riboflavin deficiency, but it did not appear to have caused any mortalty in this flock. The "chick disease" has seldom been diagnosed by laboratories conducting routine poultry disease diagnosis services. It is evident that it is not a serious cause of loss amongst chicks, and only rarely does it appear to be responsible for chick mortality.

The theory has been advanced that "chick disease" is an early acute reaction to the agent responsible for lymphomatosis in older fowls. Whilst vast numbers of chickens in all the major poultry producing countries have been affected with lymphomatosis. very few cases of "chick disease" have been recognised. The mode of transmission, *i.e.*, *via* the hatching egg and by pen contact favour widespread dissemination and in this respect the distribution might be expected to be as wide as that of lymphomatosis. The apparent rarity of the disease in a clinically recognisable form in chicks and the frequent presence of the virus in lymphomatous fowls presents a problem requiring further study. The relationship of "chick disease" virus to typical lymphomatosis remains obscure.

It is impressive that 43·6 per cent. of field cases of lymphomatosis examined failed to reveal evidence of "chick disease" virus to either the chick inoculation or serum neutralisation tests. Further more the demonstration that typical lymphomatosis can be induced by inoculation of chicks with suspensions of lymphomatous tissues apparently free of "chick disease" virus (Asplin, 1947) indicates that this virus may not be the only factor concerned in lymphomatosis if indeed it plays any part at all in initiating the disease. The theory that "chick disease" virus is an agent of low pathogenicity, widely disseminated amongst poultry flocks, cannot be ignored. If this proves to be the case it would appear that fowls affected with lymphomatosis provide a particularly suitable medium for the multiplication of the virus.

SUMMARY

Tissue suspensions of 67 fowls affected with lymphomatosis were injected into young chicks in an attempt to detect the presence of "chick disease" virus. The virus was demonstrated in 26 (38·8 per cent.) lymphomatous fowls by this method.

Tissue suspensions of 22 lymphomatous fowls which had previously been dosed with sulphadiazine (0·5 g. per day for ten

days) failed to produce lesions of "chick disease" when injected into young chicks.

Tissue suspensions of two of 41 (4·9 per cent.) non-lymphomatous fowls were infective to chicks.

The sera of all fowls whose tissues were positive to the chick inoculation test as well as the sera of six out of 30 lymphomatous fowls negative to chick inoculation test showed evidence of neutralising antibody.

Thirty-nine lymphomatous fowls were examined by both methods; 17 (43·6 per cent.) were negative to both tests.

Twenty-one non-lymphomatous fowls were examined by both methods: seven were positive to one or both tests, 14 were negative to both. It should be noted that this group contained some fowls which had been obtained from flocks in which there had been a history of lymphomatosis.

The lesions produced in chicks by all virus strains could be prevented by dosing with sulphadiazine.

It is suggested that "chick disease" is caused by a virus of low pathogenicity which is widely distributed in poultry flocks and which may not be the cause of lymphomatosis.

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OBSERVATIONS ON THE AETIOLOGY OF LYMPHOMATOSIS

III.—THE DEVELOPMENT OF LYMPHOMATOSIS IN CHICKENS FREE OF THE "CHICK DISEASE" VIRUS

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INTRODUCTION

Lymphomatosis has been the subject of intensive study in many countries during the past 20 years. Early papers dealt mainly with the clinical and pathological features of this disease complex and of its apparent spread as the result of introduction of new stock or hatching eggs into hitherto uninfected flocks. The view was frequently expressed that lymphomatosis was the result of infestation with intestinal parasites or that some nutritional deficiency was responsible. Both these theories have been disproved and no longer warrant serious consideration. Numerous investigators were successful in reproducing typical cases of lymphomatosis by the inoculation of chickens with whole blood or tissue suspensions of infected fowls. Opinions differed as to whether one or more distinct aetiologic agents were responsible for all the manifestations of the so-called avian leukosis complex.

An important step forward in elucidating the problem appeared to have been made when Blakemore (1939, 1941) reported the demonstration of a filtrable agent in the tissues of lymphomatotic fowls, which induced in young chickens lesions which had not previously been reported and which were quite unlike those of typical lymphomatosis. In baby chicks the heart was enlarged, whitish in colour and the myocardium had a smooth but irregular surface. The pericardial sac occasionally contained a yellow serous fluid. The liver showed numerous small grey foci scattered throughout the organ and sometimes interspersed with petechial haemorrhages. In some specimens there was destruction of liver tissue. Other organs were sometimes similarly affected, but rarely to the same extent. This acute response in chicks is hereafter in this report referred to as "chick disease."

Blakemore (1939) considered the possibility that there were two entirely different agents but that only one was present in the injected material, on the grounds that the original donor tissue was obtained from typical cases of neurolymphomatosis, that on study of chick tissues in all stages of the experimentally induced disease a transition from an inflammatory to a lymphomatous response could be observed, and that some of the survivors of the disease in young chicks later developed lesions typical of lymphomatosis. The theory was advanced that this acute disease in young chicks was produced by the agent responsible for lymphomatosis in older chickens and that lymphomatosis represented a chronic stage of an acute infection.

Confirmation of the presence of the virus responsible for "chick disease" in the tissues of field cases of lymphomatosis was obtained by Glover (1940)

who obtained a strain from a turkey affected with neurolymphomatosis and by Asplin (1944, 1945) from chickens.

Asplin (1945) in limited trials demonstrated the " chick disease " agent in spontaneous cases of lymphomatosis gathered from four flocks in Michigan and Massachusetts. It was not demonstrated in tissues of six lymphomatous chickens of the flock of the Regional Poultry Research Laboratory, U.S. Department of Agriculture. This is of particular interest since the laboratory stock have been maintained in strict quarantine since the establishment of the laboratory in 1939, when hatching eggs provided the foundation stock. Whilst lymphomatosis is prevalent in this flock, no other infectious diseases with the exception of caecal coccidiosis have appeared.

Peculiar difficulties attend the experimental study of lymphomatosis. In its typical form it has a long incubation period during which control of the fowls' environment to provide adequate isolation is not readily attained. There are no means of positively determining the freedom from lymphomatosis of the experimental stock nor of obtaining uniformly susceptible stock. The proportion of inoculated chickens which ultimately develop lymphomatosis is variable and is often small. *Post-mortem* lesions of lymphomatosis vary from massive tumours involving many organs and gross enlargement of peripheral nerves to scattered microscopic lymphomatous foci whose significance from a diagnostic viewpoint is doubtful. No direct visual means exist for the demonstration of virus particles or inclusion bodies.

While it is apparent that " chick disease " virus is frequently associated with field outbreaks of lymphomatosis, evidence has been obtained that it cannot be demonstrated in some cases. The experimental results reported here indicate that lymphomatosis can be induced in the apparent absence of " chick disease " virus.

ABSENCE OF CHICK DISEASE IN OUTBREAKS OF LYMPHOMATOSIS

Experiment 1

A previous report indicated that fowls apparently free of " chick disease " virus could be obtained from field outbreaks of lymphomatosis. The scope of the first experiment was to identify such a fowl.

The donor chicken which provided inocula for Experiment 1 was a 51-day-old R.I.R. pullet, killed when showing symptoms of neurolymphomatosis. *Post-mortem* examination revealed gross enlargement of numerous peripheral nerves, histologically typical of lymphomatosis. (The strain of lymphomatosis agent obtained from this fowl is referred to as Strain A.) Nerve and liver tissues were removed, ground with sand and suspended in sterile broth. The tissue suspension was immediately injected intraperitoneally into 15 seven-day-old R.I.R. chicks, each receiving a dose of 0·5 ml. During the second week after inoculation six chicks were killed and examined for evidence of " chick disease." Tissue suspensions from

these chicks were passaged through two further groups of young chicks with negative results. The remaining nine chicks were kept until five months later when all survivors were killed. The results are indicated in Table I.

It will be seen that in this experiment although no evidence of " chick disease " virus was present in the chicks killed one to two weeks after inoculation, three out of nine chicks kept for 56 to 160 days showed typical lesions of lymphomatosis. Three further serial transmissions of tissues of the lymphomatous fowl killed 88 days after infection failed to reveal evidence of " chick disease " virus.

TABLE I

Age when killed	*No.*	*Lesions*
Days		
14	2	No evident lesions
17	2	
21	2	
56	1	Neurolymphomatosis
88	1	Visceral and neurolymphomatosis
160	1	Visceral and neurolymphomatosis
160	6	No evident lesions

TREATMENT WITH SULPHADIAZINE

Experiment 2

Asplin (1946) demonstrated that " chick disease " could be effectively treated with certain sulphonamides. The object of Experiment 2 was to determine if sulphadiazine administered during the four-week period immediately following inoculation had any influence upon the ultimate incidence of lymphomatosis induced as a result of inoculation with Strain A and also to investigate the presence or absence of " chick disease " virus in chickens inoculated with this strain.

The donor chicken supplying strain A infective tissue was the 88-day-old R.I.R. pullet of Experiment 1 which was killed when showing symptoms of paralysis. *Post-mortem* examination revealed widespread lesions of neural lymphomatosis associated with a lymphomatous ovarian tumour. Tumour and nerve tissue were removed, treated in the same manner as in Experiment 1, and injected into 80 seven-day-old R.I.R. chicks. These chicks were divided into three groups, A, B and C. Further groups, D, E, F and G, served as uninjected contact and normal controls.

Group A consisted of 15 chicks which were kept for two weeks following injection and were then killed and examined for evidence of " chick disease."

Group B consisted of 32 chicks which received sulphadiazine mixed as an ingredient of the food at a level of 0·5 per cent. It was

available continuously during the 28-day period immediately following inoculation.

Group C consisted of 33 inoculated chicks which received no treatment with sulphadiazine.

(Groups B and C were raised and housed throughout their lives in separate cages but in the same room.)

Group D consisted of 30 uninoculated chicks. They were killed at 21 days of age and examined for lesions of " chick disease."

Group E consisted of 28 uninoculated chicks. They received treatment with sulphadiazine in the same manner as Group B.

Group F consisted of 30 uninoculated chicks which received no treatment.

TABLE II

Group	*Exposure*	*Treatment*	*No. of chickens*	*Chick disease*	*Lymphomatosis*	
					No. of cases	*Per cent.*
A	Injected, Strain A	Killed at three weeks old	15	0	0	0
B	"	Sulphadiazine	32	—	14	43·8
C	"	None	33	—	13	39·4
D	None	Killed at three weeks old	30	0	0	0
E	Contact	Sulphadiazine	28	—	3	10·7
F	Contact	None	30	—	5	16·6
G	None	None	791	—	0	0

Groups E and F were housed in separate brooders but in the same room with the inoculated chicks of Groups B and C for the four-week period immediately following inoculation, *i.e.*, during the period when sulphadiazine treatment was administered. They were then removed from contact and reared apart from Groups B and C.

Group G, representing a healthy control group, consisted of 791 chickens raised in the laboratory flock for stock replacement.

All chickens of Groups A to F were subjected to *post-mortem* examination, particular search being made for lesions of " chick disease " or lymphomatosis. As a routine measure specimens of liver, heart muscle and peripheral nerve were taken for histological examination. All ailing fowls in the flock comprising Group G were subjected to autopsy. The experiment was terminated after 317 days. The incidence of lymphomatosis and the chick disease is shown in Table II.

The table shows that none of the chicks killed 14 days after injection were affected with lesions of " chick disease " neither were any of the uninjected controls killed at the same time. Direct examination was not possible on chicks of the other groups. However, there was no indication of sickness and no deaths occurred in groups B, C, E and F during the first 15 days of the experiment.

No attempt has been made to classify cases of lymphomatosis under headings of neural, visceral, or mixed neural and visceral forms. The neural form predominated in the early cases ; later mixed neural and visceral forms were frequent and from the seventh month onward visceral lymphomatosis tended to predominate. No cases of osteopetrosis or ocular lymphomatosis were encountered, nor was there any evidence of erythro- or granuloblastosis.

Treatment with 0·5 per cent. sulphadiazine for four weeks had no influence upon the ultimate incidence of lymphomatosis either in the injected or contact groups of chickens. Analysis of the results did not reveal that sulphadiazine had any influence upon the age at which lymphomatosis appeared.

During the year in which this experiment was carried out 791 chickens of the same strain (Group G) were raised in the laboratory flock for stock replacement and for experimental studies. No macroscopic evidence of either lymphomatosis or " chick disease " was found in any of these fowls. It is therefore clear that the development of lymphomatosis as a result of exposure by contact to infected chickens (Groups E and F) was not the result of infection in the experimental stock.

Examination for " Chick Disease " Virus

Chick Inoculation Tests

Table II showed that none of the 15 injected chicks nor the 30 healthy stock chicks killed at three weeks old had lesions of " chick disease." The remaining chickens were killed when obviously ill or at intervals until the experiment was finally terminated at 317 days. In order to examine further for the presence of " chick disease " virus tissues were removed at *post-mortem* examination for chick inoculation tests by the usual method.

A donor fowl was recorded as negative when none of the group of injected chicks showed evidence of chick disease. Whilst it was not possible to examine all fowls of Experiment 2 in this manner, a representative number of lymphomatous and apparently normal birds from each group was tested. The results are summarised in Table III.

A total of 67 fowls were examined for " chick disease " virus by direct chick inoculation test. None of 37 fowls previously injected with Strain A, 18 of which showed gross lesions of lymphomatosis, revealed evidence of " chick disease " virus. One only of the 24 contact fowls (Group E) killed at the age of 302 days, *i.e.*, 267 days after contact with injected chicks was broken, gave a positive result. This fowl was in good condition and showed no macroscopic evidence of disease when killed. The presence of " chick disease " virus in this single fowl which had not been injected with lymphomatous material, but had received treatment with sulphadiazine may possibly represent secondary infection with this virus towards the end of the experiment.

TABLE III
CHICK INOCULATION TESTS

Group	Exposure	Treatment	No. of fowls tested	Donors			
				Lympho-matous		Non-Lym-phomatous	
				Chick disease virus			
				+	—	+	—
B	Injected, Strain A	Sulphadiazine	17	0	8	0	9
C	„	None	20	0	10	0	10
E	Contact	Sulphadiazine	11	0	2	1	8
F	Contact	None	13	0	3	0	10
G	None	None	6	0	0	0	6
			67	0	23	1	43

Serum Neutralisation Tests

The results of testing the sera of 22 fowls of Experiment 2 for serum neutralising antibodies by the method described in a previous paper are shown in Table IV.

The sera of eight fowls having gross lesions of lymphomatosis were tested. One of these, B.1125, had a neutralising effect on more than 100 chick infective doses. Of the remaining 14 sera from non-lymphomatous fowls, two neutralised 1,000 and 10,000 M.I.D. of " chick disease " virus respectively. The serum having the greater neutralising effect was obtained from a member of Group E which contained the only fowl tested whose tissues were shown to contain " chick disease " virus by direct chick inoculation test. The sera of 15 out of 19 injected or incontact fowls showed no evidence of neutralisation ; of the remaining four fowls whose sera possessed neutralising property, three had no gross evidence of lymphomatosis.

TREATMENT OF A FIELD OUTBREAK OF LYMPHOMATOSIS WITH SULPHADIAZINE AND OBSERVATIONS UPON THE PRESENCE OF " CHICK DISEASE " VIRUS IN THE FLOCK

Experiment 3

The experimental stock were obtained from a field outbreak of lymphomatosis. The practice followed on the supplying farm was the purchase of 1,600-day-old pullets from accredited flocks, every eight weeks. The chicks were brooded in outdoor brooders and reared in fold units. All pullets at 20 weeks old were sold for distribution in small groups mainly to domestic poultry keepers. Owing to the difficulty in obtaining sufficient numbers from individual breeders most eight-week groups consisted of chicks from several sources. This practice had been followed for nearly two years with negligible losses from lymphomatosis. In November, 1943, 1,600

TABLE IV

SERUM NEUTRALISATION TESTS

Group and fowl No.	*Exposure*	*Treatment*	*Lesions*	*Serum/virus titre*	*Neutralisation (M.I.D.)*
B.496	Injected, Strain A	Sd.	N	10^{-4}	0
B.1125	„	Sd.	N	$<10^{-2}$	>100
C.924	„	None	N	10^{-5}	0
Control				10^{-4}	
C.1596	Injected, Strain A	None	N	10^{-4}	0
C.1327	„	None	—	10^{-3}	10
B.1349	„	Sd.	N + V	10^{-4}	0
Control				10^{-4}	
B.51	Injected, Strain A	Sd.	N	10^{-5}	10
B.58	„	„	—	10^{-5}	10
C.89	„	None	V	10^{-5}	10
F.1735	Contact	„	—	10^{-5}	10
Control				10^{-6}	
B.1135	Injected, Strain A	Sd.	—	10^{-5}	100
B.1142	„	„	—	10^{-4}	1,000
C.1325	„	None	—	10^{-6}	10
C.1346	„	„	—	10^{-7}	0
Control				10^{-7}	
E.1651	Contact	Sd.	V	10^{-7}	0
E.1666	„	„	—	10^{-6}	10
F.1730	„	None	—	10^{-7}	0
G.2120	None	„	—	10^{-5}	100
Control				10^{-7}	
E.1608	Contact	Sd.	—	10^{-2}	10,000
F.1702	„	None	—	10^{-5}	10
G.2126	None	„	—	10^{-6}	0
G.2128	„	„	—	10^{-5}	10
Control				10^{-6}	

Sd. = Sulphadiazine
N = Neurolymphomatosis
V = Visceral lymphomatosis
— = No lymphomatous lesions

pullet chicks were obtained from a breeder who had not previously supplied stock. About 20 per cent. died within the first two weeks of life from undiagnosed cause. At six to eight weeks there was a small outbreak of caecal coccidiosis which was controlled. Cases of lameness, wing paralysis, and ataxia were seen for some time before specimens were sent for diagnosis. The presence of neural and visceral lymphomatosis was confirmed by *post-mortem* examination.

On March 11th, 1944, 108 chickens were obtained for experimental purposes. They represented a random sample of the affected flock, some of which were showing evidence of incipient lymphomatosis. The chickens were divided into two groups, T and U of 55 and 53 respectively. The chickens of Group T were dosed daily for 20 days with sulphadiazine suspended with tragacanth and administered by mouth, each receiving a daily dose equivalent to

0·5 g. per kg. body weight. Sulphadiazine significantly checked the growth rate but no deaths occurred during the period of treatment. Group U served as an untreated control. The incidence of lymphomatosis in the 12-month period following treatment (Table V) shows that four-month-old chickens obtained from a flock affected with lymphomatosis derived no benefit from treatment with sulphadiazine under the conditions of the experiment.

TABLE V

INCIDENCE OF LYMPHOMATOSIS IN SULPHADIAZINE TREATED AND UNTREATED CHICKENS FROM A FIELD OUTBREAK OF LYMPHOMATOSIS

Group	*No. of fowls*	*Treatment*	*Incidence of lymphomatosis*	
			Actual	*Per cent.*
T	55	Sulphadiazine	17	30·9
U	53	None	14	26·4

An effort was made to demonstrate the presence of " chick disease " virus in both treated and untreated groups by (*a*) direct chick inoculation tests, and (*b*) serum neutralisation tests. The results are indicated in Table VI.

Chick inoculation and serum neutralisation tests for evidence of " chick disease " virus were made on 25 fowls from this field outbreak. The virus was not found by either test from 19 fowls examined during the first 196 days of the experiment. During the period between the 233rd and 252nd day, the tissues from four out of six fowls examined by chick inoculation test gave positive results ; the sera of two of these possessed significant neutralisation titres. Three of the four " chick disease " positive fowls had received treatment with sulphadiazine during the first 28 days of the experiment. Since there was no direct evidence that " chick disease " virus was present in the fowls at the time of dosing, it cannot be concluded that the treatment had failed to remove it. Although the fowls of this experiment were housed apart from all others, the facilities available did not provide conditions of isolation sufficient to ensure against all possibility of the introduction of secondary infections. It would seem that the most probable explanation of the appearance of " chick disease " virus was that it entered as a secondary invader between 196th and 233rd day of the experiment.

DISCUSSION

Experiments were carried out in an effort to determine the validity of the theory that " chick disease " is an acute reaction caused by the agent responsible for lymphomatosis. The results, whilst not conclusive, present evidence that this is not so.

TABLE VI

EXAMINATION OF FOWLS OF EXPERIMENT 3 FOR THE "CHICK DISEASE" VIRUS

Fowl number	*Date of death*	*Sulphadiazine treatment*	*Lesions*	*Chick inoculation test*	*Serum neutralisation M.I.D.*
A.738	13. 3.44	No	N	—	10
2004	18. 3.44	No	N	—	100
2015	18. 3.44	No	N	—	0
A.747	12. 3.44	No	N	—	N.T.
2710	11. 4.44	No	N	—	N.T.
2719	26. 4.44	No	N + V	—	N.T.
2720	16. 5.44	No	H	—	N.T.
2781	22. 6.44	Yes	H	—	10
Y.10	27. 6.44	Yes	V	—	0
2738	30. 6.44	No	N + V	—	10
2740	10. 7.44	No	N	—	N.T.
2763	13. 7.44	Yes	H	—	0
2795	18. 7.44	Yes	V	—	0
2745	14. 8.44	No	I	—	10
2754	26. 8.44	No	N	—	N.T.
2779	29. 7.45	Yes	H	—	0
2721	25. 8.45	No	I	—	0
2773	4. 9.44	Yes	N + V	—	0
2709	24. 9.44	No	V	—	100
2633	31.10.44	Yes	V	+	N.T.
2729	6.11.44	No	I	—	10
2772	9.11.44	Yes	I + V	+	10,000
2723	15.11.44	No	N + V	+	10,000
2632	16.11.44	Yes	N.V.I.	+	N.T.
2624	19.11.44	Yes	H	—	N.T.

N = Neurolymphomatosis
I = Ocular lymphomatosis
N.T. = Not tested
V = Visceral lymphomatosis
H = No evidence lymphomatosis

It has been demonstrated that "chick disease" can be effectively treated with sulphadiazine. Treatment of chickens infected with lymphomatosis (strain A) for four weeks immediately following injection had no effect upon the incidence of lymphomatosis occurring during the following 317-day period. Neither had sulphadiazine any effect upon the ultimate incidence of lymphomatosis when administered to four-month-old chickens from an infected flock.

An examination was made for "chick disease" virus in chickens which had been inoculated with or in contact with lymphomatosis infected chickens. Inoculated chicks killed two weeks after injection showed no evident lesions of "chick disease." The incidence of typical lymphomatosis in 65 chickens which survived longer than four weeks after injection was 41·5 per cent. (27 cases). No evidence of the presence of "chick disease" virus was obtained by the injection of tissue suspensions from 39 strain A inoculated fowls into young chicks. Five lymphomatous and 19 non-lymphomatous incontacts were examined by the same method. The tissues of one fowl only gave a positive result. No gross or microscopic lesions of

lymphomatosis were present in this fowl which was killed 295 days after the commencement of the experiment.

The examination of injected and incontact fowls for the presence of serum neutralising antibodies gave similar results. The sera of eight lymphomatous chickens were tested; one only appeared to possess a significant level of neutralising antibody. The sera of three out of eleven non-lymphomatous chickens exposed to strain A virus possessed neutralising property against "chick disease" virus. A similar examination was made to detect evidence of "chick disease" virus in the tissues of fowls obtained from a field outbreak of lymphomatosis. Nineteen fowls were examined during the first 196 days under observation; none showed evidence of "chick disease" virus. During the period 233 to 252 days of observation six fowls were examined, four gave positive results.

In the experiments with both naturally and artificially infected lymphomatous fowls, "chick disease" virus was demonstrated towards the end of the observation period after a preliminary series of negative results. It might be argued that the "chick disease" virus was at first in some way masked and non-antigenic, and that later some event occurred which served to unmask it. The possibility that secondary infection with the "chick disease" virus took place cannot be excluded.

SUMMARY

The results presented indicate that lymphomatosis may occur in fowls not infected with "chick disease" virus. Treatment with sulphadiazine of chickens artificially and naturally infected with lymphomatosis did not influence the course of the disease.

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STUDIES ON *STREPTOCOCCUS AGALACTIAE* INFECTION

I.—INFECTION OF THE CHICK-EMBRYO AND MOUSE

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INTRODUCTION

Numerous attempts have been made to produce mouse-virulence in strains of *Str. agalactiae* in order that chemotherapeutic experiments could be carried out against this organism. Kaplan *et al.* (1942) appear to be the only workers who have reported successful results, but they carried out no passage experiments.

INFECTION OF THE CHICK-EMBRYO

Because of the generally accepted view that *Str. agalactiae* is avirulent for the mouse, we tried to establish infection in the chick-embryo. We had received several strains of *Str. agalactiae* from Dr. A. D. McEwen in October, 1943. They were kept in cooked-meat medium until April, 1944, when cultures were dried for reference, and 0·2 c.c. amounts of 1 : 5 dilutions of 24-hour serum-broth cultures were inoculated on to the chorio-allantois of chick-embryos. Most of the embryos died from streptococcal septicaemia; one strain (A4) was selected for passage as it appeared slightly more virulent than the others. The livers of dead embryos contained very numerous streptococci and were the most convenient source of inoculum. Livers of three embryos were therefore removed with aseptic precautions and suspended in normal saline; before any material had sedimented this suspension was inoculated on to the chorio-allantois of three more embryos. Thirty-seven serial passages were carried out using 0·1 to 0·01 g. liver tissue for inoculating each embryo.

A number of experiments were now carried out to see if these passages had increased the virulence of the strain for the chick-embryo and also whether there were more virulent naturally occurring strains. On one occasion groups of seven embryos were inoculated on to the chorio-allantois with 0·2 c.c. of a 10^{-1} dilution of the passage strain and with eleven freshly isolated strains of *Str. agalactiae*. None of the seven embryos was killed with the passage strain but seven of the eleven freshly isolated strains each killed one or two embryos. On other occasions *Str. agalactiae* has killed chick-embryos at much higher dilutions and we have found that the susceptibility to *Str. agalactiae* infection varies considerably from week to week. In experiment II-8 (Table I) dilutions as high as 10^{-3} killed the majority of embryos but again there was little difference between the virulence of the passage strain (A4) and of recently isolated strains of *Str. agalactiae*. The egg passage strain

TABLE I

THE VIRULENCE OF VARIOUS STRAINS OF *Str. agalactiae* FOR THE CHICK-EMBRYO

Expt. No.	*Dilution of culture inoculated on to the chorio-allantois in 0·2 c.c. amounts*	*Strain*	*No. eggs*	*Deaths*			*S*†
				1st day	*2nd day*	*3rd day*	
11·8	*Average of 10^{-1}, 10^{-2}, 10^{-3}	A4 Egg-passage strain	20	18	0	0	2
		A4 Original strain	18	17	0	0	1
38	*Average of 1 : 2, 1 : 4, 1 : 8, 1 : 16	A4 Egg-passage strain	24	14	6	0	4
		Recently isolated strain 1	18	9	4	0	5
41	*Average of 10^{-0}, 10^{-1}, 10^{-2}	A4 Egg-passage strain	17	4	2	2	9
		Recently isolated strain 2	18	10	1	2	5

* There was no significant difference in the results with the various dilutions used so the results have been summarised.

† *S* = Survivors.

has, however, been used in numerous chemotherapeutic tests and has frequently been isolated afresh from control embryos. The strain is not β haemolytic ; its serological and biochemical properties, which are shown below, have been checked from time to time and have remained constant.

Serological	Gluc.	Salic.	Inul.	Raff.	Man.	Methylene Blue Milk
Group B	+	—	—	—	—	—
		Lit. Milk Slt. A.		Hippur. +		

The following description of *Str. agalactiae* infection in the 12-day-old chick-embryo is based on observations of many embryos which died following inoculation of *Str. agalactiae* on to the chorio-allantois. The great majority died within 24 hours.

In normal embryos of this age the lungs are usually pale pink, the liver a light brown or olive colour and the kidneys a pale brown-pink or olive colour. In embryos dying from streptococcal septicaemia the liver was often dark red and the kidneys dark red and swollen ; less frequently the lungs had a similar appearance and there were sometimes haemorrhages in the heart muscle.

In section, a thick layer of cocci was seen invading the ectodermal layer of the chorio-allantois (Fig. 1), which showed necrosis. There was sometimes local invasion of the mesoderm, but elsewhere it contained very few organisms ; this layer contained an increased number of eosinophilic polymorphonuclear leucocytes,

The large blood vessels in the chorio-allantois often contained a zone of streptococci lining the endothelium, but few were found amongst the corpuscles. By contrast, in embryos inoculated with a virulent group A streptococcus (Kruger strain) only a few streptococci were seen on the surface of the chorio-allantois, there were numerous cocci lying amongst corpuscles in the large blood vessels, there was less tendency to concentration on the endothelium of these vessels and there was a more generalised infection of the mesoderm.

The same general picture was seen in the organs and with both types of streptococcus multiplication was abundant. In the liver *Str. agalactiae* was concentrated in the Kupffer cells and in this organ it again tended to line the large blood vessels (Fig. 3), but *Str. kruger* was more evenly distributed (Fig. 4). Both types of streptococci were very numerous in the glomeruli of the kidney—at times forming solid masses of cocci and necrotic areas in the glomeruli (Fig. 5). Cocci were less numerous in the lungs but were numerous in the blood vessels and meninges of the brain. *Str. kruger*, but not *Str. agalactiae*, caused a focal reaction in the brain (Fig. 2).

INFECTION OF THE MOUSE

It is known that passage in one laboratory host may sometimes increase the virulence of a virus or bacterium for another laboratory host; we therefore inoculated four mice intraperitoneally with 0·5 c.c. of a 24-hour serum-broth culture of *Str. agalactiae* (A4) in December, 1944. One of the mice died within 24 hours; the remainder survived. Two groups of ten mice were then inoculated intraperitoneally with 0·5 c.c. of 24-hour serum-broth cultures of A4 and of a recently isolated strain. Of those inoculated with A4 two died within 24 hours and three within 48 hours; the remainder survived. The other strain killed only two of the ten mice and these did not die until 48 hours. Examination of smears showed that the liver, lung and spleen were richer in organisms than the heart-blood. A 50 per cent. suspension was prepared from the liver of two of the mice inoculated with A4 and 0·5 c.c. amounts inoculated into four mice; they were all dead or moribund within 24 hours with very numerous streptococci in all organs. A 50 per cent. suspension was again prepared, and 0·5, 0·25 and 0·1 c.c. amounts inoculated into groups of five mice. All the inoculated mice were dead or moribund within 24 hours and suspensions from their livers were inoculated in 0·25 and 0·1 c.c. amounts into two groups of five mice. All mice died or were moribund within 24 hours but a few coliform organisms, in addition to the numerous streptococci, developed on a blood-agar plate which had been sown with the inoculum. Nevertheless liver suspensions were prepared from this group of mice and 0·5, 0·1 and 0·25 c.c. amounts injected into three groups of five mice. The only mouse to survive was one of the five which received the smallest dose. A liver suspension (50 per cent.) was prepared and three groups of five mice inoculated

with 0·1, 0·05 and 0·01 c.c. amounts. Four of the mice receiving the smallest dose survived, but all the remainder were dead or moribund within 24 hours. Smears showed innumerable streptococci in the organs and occasional Gram-negative rods. A pure culture of streptococci was isolated from the liver of one of these mice and it had the same biochemical and serological properties as the original A4 strain.

Half a c.c. of a 24-hour serum-broth culture of this strain now killed three of five inoculated mice in 24 hours. Passage was continued from these mice using 0·1 to 0·2 c.c. doses of 50 per cent. liver suspension. After six more passages coliform organisms again contaminated the liver suspensions. The strain was again isolated in purity, and its virulence tested when grown in three different ways: 0·5 c.c. of a 24-hour serum broth culture killed five of ten mice within 24 hours; 0·5 c.c. of a 24-hour blood-broth culture killed eight of ten mice within 24 hours; and 0·5 c.c. of a culture, produced by inoculating 1 c.c. of a 24-hour serum-broth culture into 9 c.c. of broth, containing 10 per cent. serum and 1 per cent. glucose, and incubating for four hours, only killed six of ten mice.

Passage was continued using 0·2 c.c. of liver suspension from the mice that had received the blood-broth culture. This dose killed all mice in 24 hours, and after five passages the dose was reduced to 0·2 c.c. of a 1 : 8 liver suspension. After nine passages the organism was again isolated, although there was no contamination with coliform organisms. The virulence of cultures grown for 24 hours in 10 per cent. blood-broth and broth containing 2 per cent. of mouse liver was then compared. Two groups of 20 mice weighing 16 to 17 g. instead of 20 g. were used. All but one of those inoculated with liver-broth died overnight and all but two inoculated with blood-broth. Six further serial passages were carried out. The virulence of the mouse-passage strain was then compared with the original A4 strain, which had been dried *in vacuo*, and with the egg-passage strain. Twenty-four hour blood-broth cultures were used and the results are shown in Table II.

It would appear that the egg-passage may have slightly increased the virulence for the mouse and that subsequent mouse passage had produced a much greater increase in mouse virulence.

When seven more direct passages had been carried out, giving a total of 33 mouse passages, the organism was isolated and cultures dried *in vacuo*. The strain was still serologically and biochemically identical with the original strain.

In order to make certain that our stock mice did not harbour Group B streptococci a normal mouse was killed and a liver suspension prepared. This was inoculated into five mice. They were all normal the following day but their livers were examined culturally and a fresh suspension was inoculated into five more mice. This procedure was repeated until five passages had been carried out. All mice remained healthy and *Str. agalactiae* was not isolated.

TABLE II

THE VIRULENCE OF VARIOUS PASSAGE STRAINS OF *Str. agalactiae* (A4) FOR THE MOUSE

	Str. agalactiae "*original*"			*Egg passage*			*Mouse passage* 26		
	0·1 *c.c.*	0·2 *c.c.*	0·4 *c.c.*	0·1 *c.c.*	0·2 *c.c.*	0·4 *c.c.*	0·1 *c.c.*	0·2 *c.c.*	0·4 *c.c.*
Mice weighing 20 grammes	S, S, S, S, S	S, S, S, S, S	3, S, S, S, S	S, S, S, S, S	S, S, S, S, S	1, S, S, S, S	S, S, S, S, S	1, 1, 1, 2, S	1, 1, 1, 1, 2
Mice weighing 16 grammes	S, S, S, S, S	3, S, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	3, 3, S, S, S	1, S, S, S, S	1, 1, 1, 1, S	1, 1, 1, 1, 1

S survived more than 96 hours and discarded.
Five mice were inoculated in each group. The figures 1, 2 or 3 indicate that a mouse died on the first, second or third day following.

The virulence of cultures grown in 10 per cent. blood-broth, and broth containing 10 per cent. of 1 : 5 unheated mouse liver suspension was again compared on two occasions. The results are shown in Table III and it will be seen that the liver-broth culture was again more virulent than the blood-broth culture. The strain has now been used for chemotherapeutic tests, in which approximately 500 mice have died and numerous streptococci have been demonstrated in the livers of all those examined. Treated animals have survived, or died at intervals up to five days after

TABLE III

MOUSE PASSAGE 33, VARIOUS DOSES OF 18 HOUR CULTURE GIVEN INTRAPERITONEALLY

Expt. No.	*Liver Broth*			*Blood Broth*		
	0·1 *c.c.*	0·2 *c.c.*	0·4 *c.c.*	0·1 *c.c.*	0·2 *c.c.*	0·4 *c.c.*
1	1, 1, 2, 2, S	1, 1, 1, 2, S	1, 1, 1, 1, 1	S, S, S, S, S	1, 1, S, S, S	1, 1, 1, 1, 1
2	1, 1, 1, S, S	1, 1, 1, 1, 1	1, 1, 1, 1, 1	S, S, S, S, S	1, 1, 1, 1, S	1, 1, 1, 1, 1

inoculation. Subsequent work which has not been carried to completion suggests that mouse-virulence can be produced in strains of *Str. agalactiae* without preliminary passage in the chick-embryo.

Observations have been made on mice dying following the inoculation of *Str. agalactiae* (A4) and various Group A streptococci. Barnard and Todd (1940) found that mice dying following the intraperitoneal inoculation of living virulent or avirulent Group A streptococci showed cloudy swelling of the parenchymatous organs, congestion of the lungs and "slight changes" in the spleen, all of which were compatible with bacteraemia. In only one mouse which survived 40 hours was there appreciable lakeing of the blood. Both the virulent and attenuated cocci formed a layer on the surface of the liver, but in addition the virulent cocci were distributed throughout the liver substance.

Histological examination of tissues from mice dying following the intraperitoneal inoculation of *Str. agalactiae* usually showed numerous cocci on the surface of the liver, but although *Str. agalactiae* must be considered an "avirulent" streptococcus for the mouse there were, as one would expect, quite large numbers of cocci distributed throughout the liver substance, chiefly in the Kupffer cells (Fig. 6). Only occasional cocci were seen in the kidney and the lung, or lying among corpuscles in the large blood vessels. Cocci were often very numerous on the surface of the liver and in the loose areolar tissue round the kidney, adrenal, pancreas and

spleen, while there were often masses in the similar tissue of the mediastinum (Fig. 7), particularly around the large vessels where in some areas this tissue was undergoing necrosis. Cocci were also numerous in the bronchial lymph-nodes, particularly in the peripheral sinus which showed "sinus-catarrh" and early necrosis (Fig. 8). In some mice there were masses of cocci lying between the endo- and epicardium, but myocardial necrosis was not observed. Although congestion of the lungs was the only definite macroscopic change, the lungs contained fewer cocci than the liver or spleen. There was, however, slight subpleural concentration of organisms in the lungs. Cocci were fairly numerous in the meninges.

No distinct microscopic changes were seen in the liver, spleen or kidney, but there were slight haemorrhages in the adrenals. In general the number of polymorphonuclear cells observed was small, the majority of cells associated with groups of cocci being lymphocytes or large mononuclear cells.

Mice dying following the intraperitoneal inoculation of a virulent Group A streptococcus were examined and no clear difference could be observed in the distribution of cocci in the lesions produced.

It appears that the great concentration of organisms associated with the serous membranes and the areolar tissue of the mediastinum and the bronchial lymph-nodes is a characteristic occurrence following intraperitoneal inoculation. It has been observed in mice inoculated intraperitoneally with *Myco. johnei* (Francis, 1943), but not in a few mice which died following the subcutaneous inoculation of *Str. agalactiae* (A4).

COMPARISON OF VIRULENCE IN THE CHICK-EMBRYO AND MOUSE

A series of experiments was carried out to compare the virulence of the chick and mouse-passage strains with the original strain of *Str. agalactiae* (A4) and with various Group A streptococci; representative results are shown in Table IV. In experiment 90, there was no real difference in the virulence of the original mouse or egg-passage strains for the embryo, but although 0·02 c.c. of culture of the mouse-passage strain killed six of eight mice, 0·5 c.c. of the original strain, and of the egg-passage strain, failed to kill any mice.

In experiment 85, 10^{-0} and 10^{-1} dilutions of the egg-passage strain of *Str. agalactiae* (A4) killed the majority of embryos but failed to kill any mice. On the other hand, although the 089 mouse-virulent Group A strain killed mice at high dilutions and even the 089 mouse-avirulent strain killed a few mice, both these strains were of very low virulence for the chick-embryo. The Richard V and Richard G strains were respectively very lethal and slightly lethal for mice but only had about the same virulence as *Str. agalactiae* for the chick-embryo.

TABLE IV

THE VIRULENCE OF VARIOUS STRAINS OF *Str. agalactiae* (A4) AND GROUP A STREPTOCOCCI FOR MICE INOCULATED INTRAPERITONEALLY AND CHICK-EMBRYOS INOCULATED ON TO THE CHORIO-ALLANTOIS

Expt. No.	Strain	Mice: Dose c.c.	Mice: Dilution	Mice: No. of mice	Mice: Deaths on days 1	2	3	4	Mice: Survivors	Chick-embryos: Dilution of culture inoculated on to the chorio-allantois in 0·2 c.c. amounts	Chick-embryos: No. of eggs	Deaths on days 1	2	3	Survivors
90	*Str. agalactiae* (A4) — Egg-passage strain	0·5	10^{0}	8	0	0	0	0	8	Average of 1 : 1, 1 : 5, 1 : 25	23	17	5	0	1
		0·2	10^{-0}	8	0	0	0	0	8						
		0·1	10^{-0}	8	0	0	0	0	8						
	Mouse-passage strain ...	0·5	10^{0}	8	7	0	0	0	1		23	10	11	0	2
		0·2	10^{-0}	8	6	0	0	0	2						
		0·1	10^{-0}	8	1	0	0	0	7						
	Original strain	0·5	10^{-0}	8	0	0	0	0	8		25	12	6	1	6
		0·2	10^{-0}	8	0	0	0	0	8						
		0·1	10^{-0}	8	0	0	0	0	8						
85	Group A streptococci* — Egg-passage strain	0·2	10^{-0}	5	0	0	0	0	5	Average of 10^{0} and 10^{-1}	11	7	2	1	1
		0·2	10^{-1}	5	0	0	0	0	5						
	089, mouse-virulent strain ...	0·2	10^{-4}	5	5	0	0	0	0	Average of 10^{-0} and 10^{-1}	8	0	4	2	2
		0·2	10^{-5}	5	3	1	0	0	1						
		0·2	10^{-6}	5	1	1	0	0	3						
	089, mouse-avirulent strain...	0·2	10^{-0}	5	3	0	0	1	1	Average of 10^{-0} and 10^{-1}	8	0	1	0	7
		0·2	10^{-1}	5	0	0	0	0	5						
	Richards V, mouse-virulent	0·2	10^{-4}	5	2	2	1	0	0	Average of 10^{-0} and 10^{-1}	12	9	1	1	1
		0·2	10^{-5}	5	0	0	1	0	4						
		0·2	10^{-6}	5	0	0	0	0	5						
	Richards G, mouse-avirulent	0·2	10^{-0}	5	2	2	0	1	0	Average of 10^{-0} and 10^{-1}	11	8	2	1	0
		0·2	10^{-1}	5	0	3	0	0	2						
	Str. kruger strain	0·2	10^{-5}	5	5	0	0	0	0	Average of 10^{-2} and 10^{-3}	12	5	2	1	4
		0·2	10^{-6}	5	2	3	0	0	0	„ 10^{-4} „ 10^{-5}	12	0	3	1	8
		0·2	10^{-7}	5	1	1	0	0	3						

* I am indebted to Dr. E. W. Todd who kindly supplied the Group A streptococci, with the exception of the Kruger strain, which is used by Dr. A. R. Martin for routine chemotherapeutic tests in our laboratories.

SUMMARY

A strain of *Str. agalactiae* was carried through 37 direct passages in the chick-embryo, and 33 in the mouse.

The nature of the infection in these hosts is described and compared with infection by Group A streptococci.

The virulence of various strains of *Str. agalactiae* and of Group A streptococci was compared in the chick-embryo and mouse. There was no correlation between virulence for these two hosts.

ACKNOWLEDGMENTS

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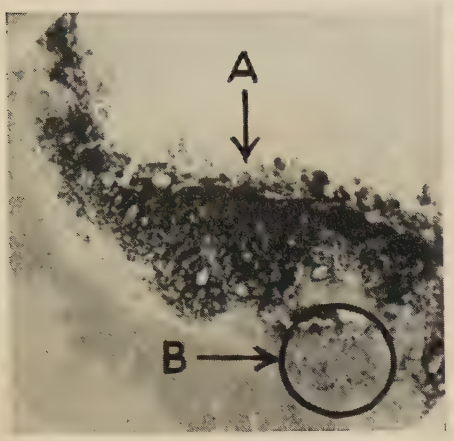

FIG. 1

Embryo 139. Dead 24 hours after inoculation. Chorio-allantois: masses of *Str. agalactiae* in epithelium (A). Some have invaded the mesoderm and others can be seen in a blood vessel (B). Gram (394/46) × 375.

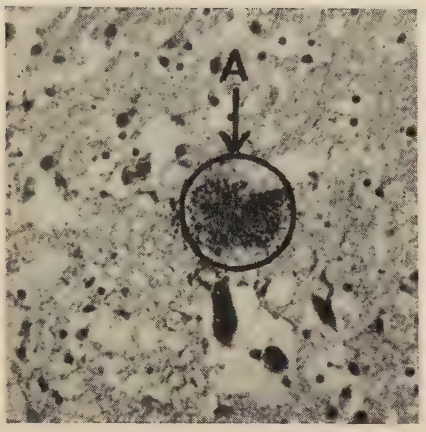

FIG. 2

Embryo 59. Dead 48 hours after inoculation. Brain: considerable spongy breakdown of tissue in cerebrum and scattered foci of softening around embolic clumps of Group A streptococci, one of which is shown at A. Numerous foci were also present in the cerebellum. H. & E. (399/46) × 375.

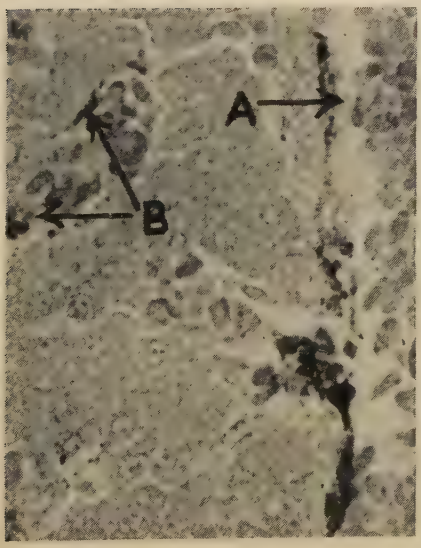

FIG. 3

Embryo 139. Dead 24 hours after inoculation. Liver: numerous *Str. agalactiae* lining a central vein (A), groups can also be seen in Kupffer cells (B). Gram (549/46) × 750.

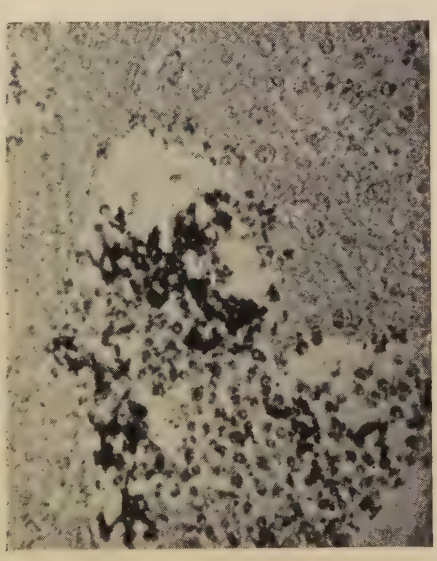

FIG. 4

Embryo 57. Dead 24 hours after inoculation. Liver: numerous group A streptococci are in the lumen of a central vein, there is no layer adherent to the endothelium. Gram (551/46) × 750.

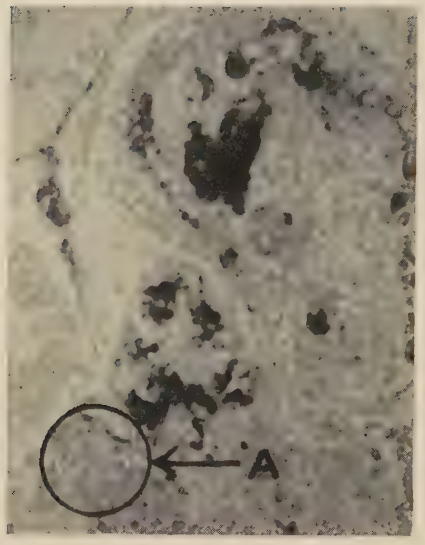

FIG. 5

Embryo 139. Dead 24 hours after inoculation. Kidney: masses of *Str. agalactiae* in glomerulus. A few cocci can be seen lining a blood vessel (A) but none can be seen lying between the corpuscles. Gram (548/46.) × 375.

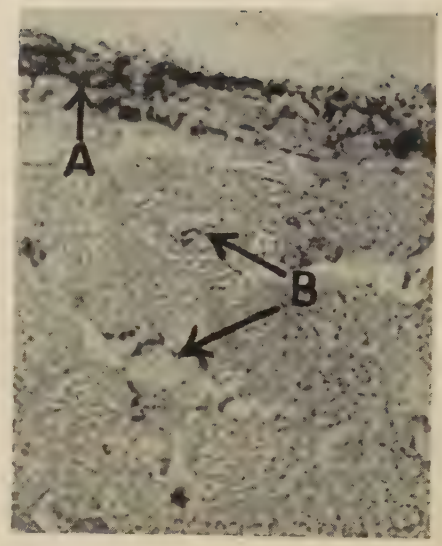

FIG. 6

Mouse 614. Dead 24 hours after inoculation. Liver: masses of *Str. agalactiae* on peripheral surface (A) and groups in Kupffer cells (B). Gram (400/46) × 750.

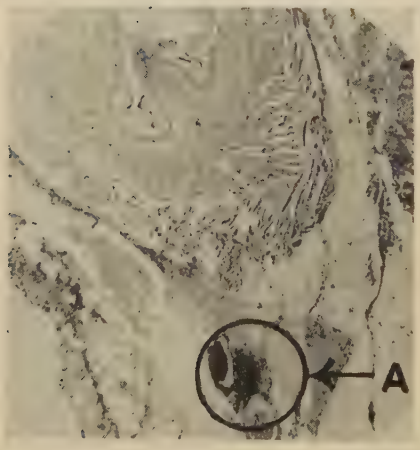

FIG. 7

Mouse 616. Dead 24 hours after inoculation. Oesophagus and mediastinal tissues: a large mass of *Str. agalactiae* can be seen at A and smaller groups to the left. Gram (391/46) × 55.

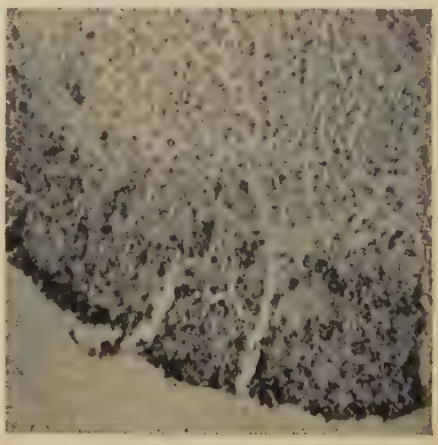

FIG. 8

Mouse 615. Dead 24 hours after inoculation. Bronchial lymph-node: numerous *Str. agalactiae* which are concentrated in the peripheral sinus and cortex. Gram (545/46) × 375.

STUDIES ON *STR. AGALACTIAE* INFECTION
II.—THE RELATIVE POTENCIES OF THE COMMON SULPHONAMIDES AND 4 : 4'DIAMINO-DIPHENYL SULPHONE IN THE CHICK-EMBRYO AND MOUSE*

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INTRODUCTION

When we began work on the chemotherapy of *Str. agalactiae* infection there were at least seven sulphonamide compounds which could be used. To compare their value in the naturally occurring disease would have been a formidable task, so other methods were adopted.

The establishment and nature of *Str. agalactiae* infection in the chick-embryo and mouse have been described by Francis and Peters (1947) and the general principles underlying chemotherapeutic research in the chick-embryo by Francis (1946).

TECHNIQUE

Chick-Embryos.—Twelve-day-old embryos, and the egg-passage strain of *Str. agalactiae* (A4) were used throughout the work. A 50 per cent. dilution of a 24-hour blood-broth culture was inoculated on to the chorio-allantois in doses of 0·2 ml.

All drugs were included in each experiment so that there was always a direct comparison between the results with the various drugs and those in the control group. Following inoculation the aperture on the top of the shell was covered with scotch tape and one hour later the various sulphonamides, suspended in 0·5 c.c. of distilled water, were inoculated into the allantoic cavity through a small hole drilled in the side of the shell. This hole was then sealed with paraffin wax. The eggs were incubated at 37° C., examined morning and evening for three days, and smears made from the liver of dead embryos. Streptococci hardly ever invade the liver when inoculated on to the chorio-allantois of a dead embryo, so if few or no streptococci were found in the liver death was assumed to be due to causes other than multiplication of organisms, *i.e.*, death was non-specific.

Mice.—Mice were given the various sulphonamides by mouth and one hour later injected intraperitoneally with 0·5 c.c. of a 24-hour blood-broth culture of the mouse passage strain of *Str. agalactiae* (A4). The mice were observed morning and evening for four days. At first smears were made from the liver of dead animals but as they almost invariably contained numerous streptococci this was discontinued.

The following survival times were given to animals dead at the intervals shown below :—

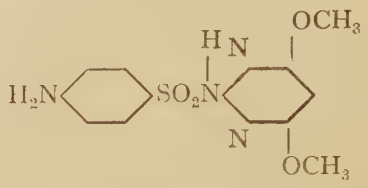
Days	...	...	1	2	3	4
Morning (hours)		...	14	38	62	86
Evening („)		...	26	50	74	98

[Survivors 120 hours]

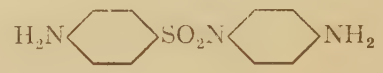
* Henceforth called sulphone.

The following drugs were used : sulphanilamide, sulphapyridine, sulphathiazole, sulphadiazine, sulphamezathine.

† Sulphadimethoxy-pyrimidine " 3445 "



4 : 4′ diamino-diphenyl sulphone



RESULTS

Chick-embryos

As already mentioned, if an appreciable number of streptococci was not found in the liver of a dead embryo, it was assumed that death was due to " non-specific " causes. Embryos which died in this way have been excluded when calculating the weighted mean specific deaths which is done in the following way. Deaths on the first day are obviously of more significance than those on later days, so the number of eggs dying on the first day are multiplied by three, those on the second day by two and those on the third day by one. The total is divided by the total number of eggs less the number dying non-specifically. This can be recognised as four minus the mean survival time in days where all survivors are given an arbitrary survival time of four days. The weighted mean as calculated above is, therefore, a satisfactory criterion to use as a measure of the response to any given dose of drug in any given experiment.

Calculation of relative potencies. The sulphonamides were given in doses of 1·25, 2·5, 5, 10 and 20 mg. The weighted mean specific deaths and the percentage survivors are shown in Table I, and Graph 1, from which it will be seen that there was a steady decrease in mean specific deaths and increase in survivors with increase in dose. Doses of 2·5 and 5·0 mg. are common to all ten experiments and in this region the weighted mean specific deaths are linearly related to the log. of the dose. For appreciably higher and lower doses the response curve flattens. The comparison of the activities of the various drugs was restricted to the linear region, *i.e.*, the 2·5 and 5 mg. doses. The number of eggs less the number of non-specific deaths varies from one experiment to another, but the differences are not great and no serious error results if it is assumed that the mean specific deaths in each experiment have equal weight ; this simplifies the analysis considerably. Sulphone is considered separately owing to its toxicity. As a statistical analysis of the data showed that the slope of the response curve did not differ signific-

† The synthesis of this drug was described by Rose and Tuey (1946).

TABLE I

THE WEIGHTED MEAN SPECIFIC DEATHS AND THE PERCENTAGE SURVIVORS OF CHICK-EMBRYOS INFECTED WITH *Str. agalactiae* (A4) AND TREATED WITH VARIOUS SULPHONAMIDES ONE HOUR LATER

Dose Mg.	*Exp. No.*	*Sulph-anilamide*	*Sulpha-pyradine*	*Sulpha-diazine*	*Sulpha-mezathine*	*Sulpha-thiazole*	*Sulphone*	*Average death-rate of all embryos receiving each dose level**	*Controls*
20	13	0·9			0·0				2·1
	14	0·0			0·0				1·3
Average		0·45			0·0			0·23 (64·9)	1·7
	51	0·8	0·0	0·6	1·0	2·0			2·1
10	52	0·0	0·4	0·6	0·0	0·0			2·4
	53	0·67	1·0	1·5	0·0	0·0			2·8
	54	0·67	0·0	0·4	0·0	0·0			3·0
Average		0·53	0·35	0·77	0·25	0·5		0·44 (49·6)	2·57
	51	0·8	3·0	0·75	0·75	0·0	2·0		2·1
	52	1·0	0·29	1·5	0·0	0·0	1·5		2·4
	53	1·0	2·5	1·33	0·67	0·75	1·25		2·8
	54	0·8	0·83	1·8	1·29	0·0	2·0		3·0
5	58a	0·6	0·0	3·0	1·5	1·5	0·75		3·0
	58b	1·25	1·8	0	3·0	1·6	1·0		3·0
	59	2·4	2·17	1·6	2·25	1·0	1·0		3·0
	60	3·0	1·67	2·0	0·75	1·2	0·0		3·0
	63a	2·2	1·5	1·5	0·0	1·0	0·0		3·0
	63b	2·25	2·0	2·8	2·0	1·5	1·5		3·0
Average		1·53	1·58	1·63	1·22	0·86	1·05	1·36 (35·9)	2·83
	51	3·0	0·25	1·2	2·0	1·0	2·0		2·1
	52	2·0	2·57	2·33	0·57	0·75	0·5		2·4
	53	3·0	3·0	2·8	1·5	2·2	1·8		2·8
	54	2·43	1·5	2·8	1·17	0·8	1·5		3·0
2·5	58a	3·0	3·0	1·2	2·75	2·0	1·0		3·0
	58b	2·33	3·0	2·4	2·6	2·0	1·0		3·0
	59	2·0	1·75	1·67	1·5	1·83	1·6		3·0
	60	3·0	2·25	2·8	2·25	2·25	2·25		3·0
	63a	3·0	1·4	1·4	0·75	0·75	0·0		3·0
	63b	2·6	2·4	2·5	2·33	3·0	2·0		3·0
Average		2·64	2·11	2·11	1·74	1·66	1·38	2·05 (19·8)	2·83
	58a	3·0	2·0	1·8	2·25	3·0	2·0		3·0
	58b	2·67	3·0	3·0	1·6	3·0	1·5		3·0
1·25	59	3·0	2·0	2·8	2·5	2·8	2·0		3·0
	60	2·4	3·0	1·6	3·0	2·2	2·0		3·0
	63a	2·0	1·0	2·0	2·0	1·67	2·3		3·0
	63b	2·83	2·67	3·0	3·0	3·0	2·5		3·0
Average		2·65	2·28	2·36	2·39	2·61	2·06	2·46 (11·3)	3·0

* Excluding sulphone. The percentage survivors at each dose-level, again excluding sulphone, are shown in brackets.

antly between drugs, the activities of the drugs can be compared directly. The mean slope came to 0·67 per twofold difference in concentration. The weighted mean specific deaths over the two doses and over the ten experiments came to : sulphanilamide, 2·8 ; sulphapyridine, 1·85 ; sulphadiazine, 1·82 ; sulphamezathine, 1·49 ; sulphathiazole, 1·26

The lower the value of the mean specific deaths the greater the activity of the drug. Sulphathiazole thus gives the highest apparent activity (excluding sulphone). The standard deviation of each mean is 0·16 from which one deduces that a difference of 0·45 between any two drugs is required for significance at the 5 per cent. level of probability.

The differences between the mean specific deaths following the use of sulphanilamide, and any other drug, divided by the slope, 0·67, is the log. to the base 2 of the ratio of the activities of the two drugs. This gives the activity of any drug in terms of sulphanilamide and these ratios are shown in Table III. A ratio of 1 : 1·59 between two drugs is required for significance at the 5 per cent. level. Sulphathiazole and sulphamezathine are about twice as active as sulphanilamide ; sulphapyridine and sulphadiazine do not differ significantly from sulphanilamide.

TABLE III

THE RELATIVE POTENCIES OF VARIOUS SULPHONAMIDES AGAINST *Str. agalactiae* INFECTION IN THE CHICK-EMBRYO AND MOUSE

	Str. agalactiae, *this study*				*Group A streptococcus*
	In eggs	*Crude potency in mouse*	*B.L.C.A. in mouse*	*Intrinsic potency in mouse*	*Marshall* et. al. (1940)
Sulphanilamide	1	1·0	1·0	1	1
Sulphapyridine	1·25	2·0	1·6	1·25	1·09
Sulphamezathine	1·84	2·8	2·3	1·22	
Sulphadiazine	1·30	8·1	4·1	2·0	
Sulphathiazole	2·33	0·71	0·4	1·8	
Sulphone	3·15	5·2	2·1	2·5	2·92
" 3445 "		1·7	5·4	0·31	

Sulphone was not included in the above analysis owing to its high toxicity. The weighted specific deaths were slightly less reliable than for the other drugs ; over all experiments they were 1·38 and 1·91 at the 2·5 mg. and 1·25 mg. dose-levels respectively and the slope of 0·53 agreed reasonably well with the slope of 0·67 for the other drugs. Allowing for the smaller doses, sulphone had a relative potency of 3·15 compared with sulphanilamide. Sulphone was thus the most potent of the drugs examined.

Toxicity of drugs. The weighted mean non-specific deaths were

calculated in the same way as the specific deaths, except that the sum of deaths on the various days (after appropriate multiplication) was divided by the total number of eggs at the start of the experiment. This figure may clearly show the toxicity of a drug, for if it killed all embryos on the first day the mean non-specific deaths would be three. The summarised results are shown in Table II, and it will be seen that the mean non-specific deaths (excluding embryos injected with sulphone) doubled as the dose increased from 1·25 mg. to 10 mg. but there was little difference between the toxicity of the various sulphonamides. At the 5 mg. dose level sulphone was nearly twice as toxic as the other compounds and could not be used at a dose of 10 mg. Sulphanilamide is recognised to be much more toxic than the other sulphonamides for the hatched chick (Bieter *et al.*, 1941; Asplin, 1944), and it is interesting that this difference was not observed in the chick-embryo.

TABLE II

THE WEIGHTED MEAN NON-SPECIFIC DEATHS OF CHICK-EMBRYOS INFECTED WITH *Str. agalactiae* AND TREATED WITH VARIOUS SULPHONAMIDES ONE HOUR LATER

Dose Mg.	*Sulph-anilamide*	*Sulpha-pyra-dine*	*Sulpha-diazine*	*Sulpha-meza-thine*	*Sulpha-thiazole*	*Sul-phone*	*Average death-rate of all embryos receiving each dose level* *	*Con-trols*
10	1·18	1·33	1·01	1·09	0·9		1·09	0·265
5	0·751	0·53	0·943	1·03	0·686	1·453	0·79	0·303
2·5	0·758	0·81	0·703	0·662	0·938	1·265	0·771	0·303
1·25	0·16	0·76	0·58	0·71	0·61	0·75	0·564	0·35

* Excluding sulphone.

Mice. The drugs were given in doses of 2, 1, 0·5 and 0·25 mg. On two occasions eighteen hours were allowed to elapse before the drugs were given and even then some gave partial protection at doses of 2 and 1 mg. In the other four experiments the drugs were given one hour after infection and the detailed results are shown in Table IV. The average survival time of all mice (105-140) receiving each dose-level of all the drugs was calculated and then plotted (Graph 2). The average survival time of all mice receiving all doses of each drug (55 mice) were then placed on this curve. The relative potencies of the drugs can be calculated from this graph but the results shown in Table III are based on a statistical analysis, from which it was shown that twofold differences in potency are required for significance (P 0·05). This graphical method was shown to be valid because the response curve was linear with log. dose and the slope did not differ significantly between drugs.

TABLE IV

THE AVERAGE SURVIVAL TIME IN HOURS AFTER THE ADMINISTRATION OF THE SULPHONAMIDES AND SULPHONE BY MOUTH FOLLOWED BY THE INTRA-PERITONEAL INOCULATION OF 0·4 C.C. OF A 24-HOUR CULTURE OF *Str. agalactiae* MICE

Time before infection	*Dose Mg.*	*Exp. No.*	*Sulphanilamide*	*Sulphamezathine*	*Sulphadiazine*	*Sulphathiazole*	*Sulphapyridine*	"3445"	*Sulphone*	*Average survival time of all mice receiving each dose level*	*Controls*
18*	2	45	14	14	16·4	14	14	20·8	35·2		
1	2	47	49·6	120	120	61·2	98·8	120	120		
1	2	48	68·4	120	120	66	94·4	120	120		
	Average		59·0	120	120	63·6	96·6	120	120	102·7	
18*	1	44	14	14	37·6	16·4	14	57·2	40·4		
1	1	44	46	82·4	87·2	29·0	35·2	42·4	90		14
1	1	45	14	75·6	120	18·8	16·4	42·4			16
1	1	47	61·2	87·2	120	40·0	44·8	79·6	120		33·2
1	1	48	101·2	82·4	120	23·6	101·2	77·6	103·6		16·4
	Average		55·6	65·5	111·8	27·9	49·4	60·5	104·5	67·9	
1	0·5	45	18·8	23·2	35·6	14·0	35·6	16·4	59·6		
1	0·5	47	23·6	54·4	120	27·2	20·8	26·0	120		
1	0·5	48	63·6	101·2	113·2	14	41·6	40·4	94·4		
	Average		35·3	59·6	89·6	18·4	32·7	27·6	92	50·8	
1	0·25	44	16·4	35·6	64·4	14·0	16·4	14·0	18·8		
1	0·25	45	14·0	16·8	61·6	14·0	14·0	16·4	42·8		
1	0·25	47	28·4	49·6	120·0	28·8	63·6	28·4	68·6		
1	0·25	48	14·0	62·0	113·2	14·0	14·0	30·8	40·0		
	Average		18·2	41	89·8	17·7	27·0	22·4	42·5	36·9	
Average survival time, all doses			42	71·5	102·8	31·9	51·4	57·6	92·2		19·9
Total number of survivors, all doses			13	30	51	6	17	20	37		0

* These results are not included in the averages.

DISCUSSION

The relative potencies of the various drugs based on the mean survival time in mice is very different from those in the chick-embryo, but this is to be expected because the "crude potency" of a drug depends as much on its pharmacology as on its antibacterial potency (Table III). We have found (unpublished results) that there are no large differences in the blood-levels produced by the various sulphonamides in the chick-embryo, but there are large differences in the mouse. Rose and Spinks (1946) carried out an accurate study in the mouse and the relative blood-level curve-areas (B.L.C.A.'s) produced by identical doses of the same drugs can be calculated from their figures: these are shown in Table III. The antibacterial or intrinsic potency of the various drugs has been calculated by dividing the relative B.L.C.A.'s into the relative crude potencies. Following the administration of single doses, the blood-concentration of the various drugs may occur at different times and the relative B.L.C.A.'s are probably not identical at different dose-levels. The method may, therefore, be less satisfactory than that adopted by Marshall, *et al.* (1940), who performed a series of experiments in which the concentrations of sulphanilamide, sulphapyridine, and sulphone, in the food of mice, were so adjusted that the same blood-levels were produced by all drugs. Using the "median survival blood concentrations" as the basis of comparison they concluded that the relative potencies of sulphanilamide, sulphapyridine and sulphone against *Str. pyogenes* infection in mice were, respectively, 1, 1·09 and 2·92. It will be seen, therefore, that the method we have used to calculate antibacterial potency in the mouse has given results in quite close agreement with those obtained by Marshall *et al.* (1940).

The drug "3445" was found to be very active against Group A streptococcal infection in mice by Gage *et al.* (1946). We have confirmed this observation and conclude, therefore, that its low activity against *Str. agalactiae* infection is due to a specific difference between these organisms.

A statistical comparison of the egg and mouse experiments shows that the accuracy is approximately the same, with equal numbers of eggs and mice, when doses giving a linear response curve are used.

SUMMARY

The relative potencies of various sulphonamide drugs and 4 : 4′diaminodiphenyl sulphone against *Str. agalactiae* infection have been compared in the mouse and chick-embryo.

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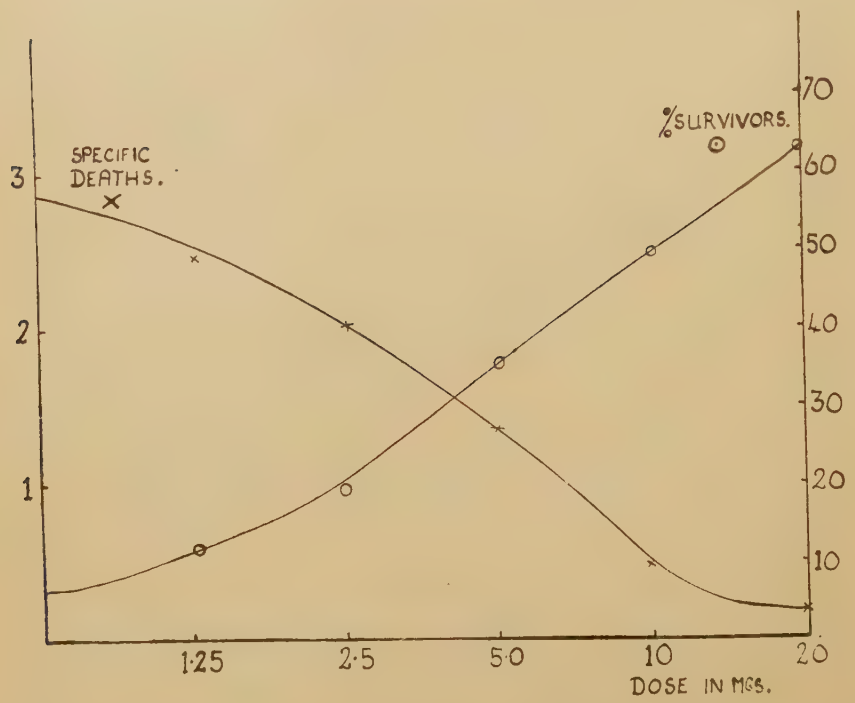
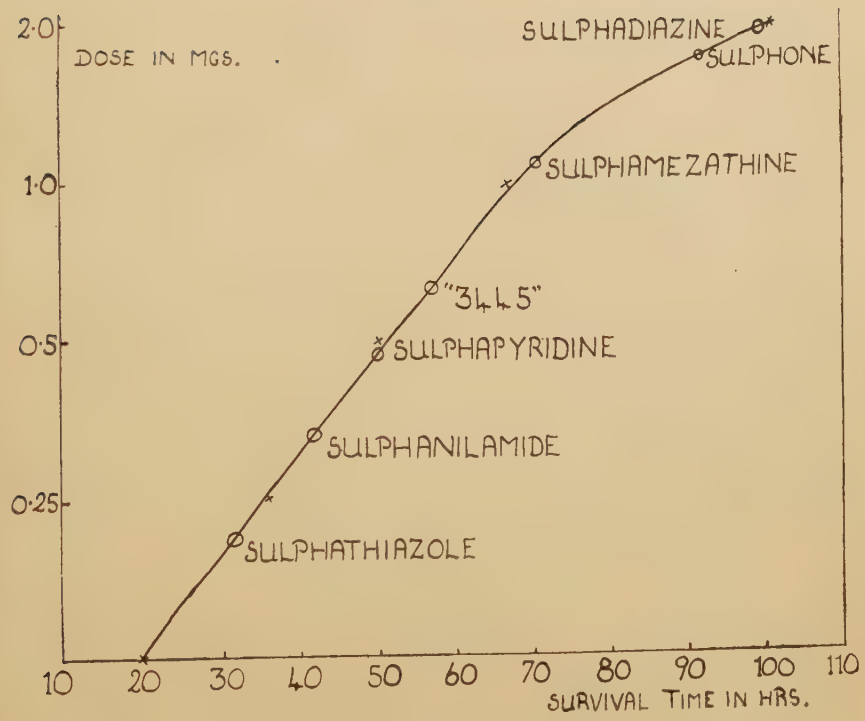


GRAPH I.

Weighted mean specific deaths and percentage survivors of chick-embryos inoculated on to the chorio-allantois with *Str. agalactiae* (A4) and into the yolk sac with various doses of the sulphonamides.



GRAPH II.

The survival time of mice inoculated intraperitoneally with *Str. agalactiae* (A4) and treated orally with various doses of the sulphonamides.

The curve is obtained by averaging the survival time of all mice inoculated with each dose level (x); the survival time of all mice receiving each drug is then averaged and placed on this curve (o). (Table IV.)

The relative activity can be calculated from the vertical distance between any two drugs.

STUDIES ON *STR. AGALACTIAE* INFECTION

III.—THE RELATIVE POTENCIES OF SULPHANILAMIDE, SULPHAPYRIDINE, 4 : 4′-DIAMINO-DIPHENYL SULPHONE AND VARIOUS SOLUBLE DERIVATIVES AGAINST STR. AGALACTIAE INFECTION IN THE CHICK-EMBRYO AND MOUSE

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INTRODUCTION

The relative potencies of the common sulphonamides against *Str. agalactiae* infection in the chick-embryo and mouse were determined by Francis, Peters and Davies (1947). Francis (1947a, 1947b) found that sulphapyridine, sulphathiazole and sulphadiazine produced lower blood levels than sulphanilamide in the bovine. Sulphamezathine, sulphamerazine, sulphadiazine and 4 : 4′-diaminodiphenyl sulphone* produced higher blood levels than sulphanilamide, but McEwen, *et al.* (1941) found that the levels of sulphone in milk were only about one third of those in the blood, and this was confirmed by Francis (1947a). We have carried out no experiments with the other sulphonamides but they all pass less readily into the cerebro-spinal fluid than does sulphanilamide. It seems probable therefore that, in contrast to sulphanilamide, they will produce lower blood than milk-levels and this, combined with their greater cost and only slightly greater antibacterial action against *Str. agalactiae,* renders it improable that they will ever be used for the treatment of mastitis by oral dosing.

Any of the sulphonamides can be injected into the udder as suspensions in oil or water, but as it is not always easy to produce satisfactory preparations, a number of soluble derivatives were examined. The sodium salts of the sulphonamides are rapidly absorbed from the udder (Francis 1946b) and their high alkalinity and consequent irritant action renders them unsuitable for injection. The sodium salt of sulphacetamide is, however, neutral and non-irritant when injected into the udder and in two experiments we found it to be fairly persistent. The potency of this drug and various soluble derivatives of the sulphonamides, which are relatively non-irritant when injected into the udder, were compared.

TECHNIQUE

The technique described by Francis and Peters (1947) was adopted, except that in one series of experiments the drugs were given subcutaneously to mice. The doses of all the solubilised derivatives were calculated on the weight of the parent sulphon-

* Henceforth called sulphone.

amide in the organic complex. Thus throughout this paper a dose of 1 mg. of sulphanilamide E.O.S. (etc.) means that the dose contained 1 mg. of sulphanilamide. There is therefore always strict comparison with the parent compound.

RESULTS

The following drugs were used : sulphanilamide ; sulphanilamide E.O.S.* ; sulphanilamide L.S.F. (sulphanilamide lactoside sodium formaldehydesulphoxylate) ; soluseptasine (disodium para-(γ-phenylpropylamino) benzenesulphonamide-α, γ-disulphonate 20 per cent. w/v in distilled water.

Sulphapyridine ; solupyridine (disodium 2-[p-(α-phenylpropylamino)- benzenesulphonamide] pyridine α : γ-disulphonate, 45 per cent. w/v solution).

Sulphone ; sulphone E.O.S. ; sulphacetamide.

Mice

The results following oral and subcutaneous administration of drugs, after the intraperitoneal inoculation of mice with *Str. agalactiae* (A4), are shown in Tables I and II, and graphically in Figs. 1 and 2 ; statistical analysis shows that twofold differences in potency between any two drugs are required for significance (P 0·05), and the calculated differences are shown in Table IV.

Chick-embryos

In two relatively small series of experiments it was found that the E.O.S. derivatives of sulphanilamide and sulphone were rather less active than the parent compounds and that sulphanilamide L.S.F. gave no protection at all. Sodium sulphacetamide was also inactive at the dose used (Table III).

DISCUSSION

The different results obtained with the two routes of inoculation in the mouse and in the chick-embryo may appear discrepant, but it should be remembered that the complex organic derivatives of the sulphonamides are not active until the free drug has been dissociated. The degree of dissociation may vary under different conditions and in the mouse different rates of absorption from the intestine and the subcutaneous tissues, as well as excretion by the kidney, all influence the crude potency. Some facts are, however, quite clear cut.

Sulphacetamide was not used in the mouse because it is poorly absorbed, but it was clearly of very low potency in the chick-embryo, an average dose of 6·6 mg. giving no protection. Sulphanilamide L.S.F. was quite non-toxic to the chick-embryo, but again quite

* The abbreviation "E.O.S." is not strictly correct but has become established in the literature.

TABLE I

THE SURVIVAL TIME IN HOURS OF MICE GIVEN SULPHANILAMIDE, SULPHONE AND SULPHAPYRIDINE PER OS AS THE FREE DRUG AND SOLUBILISED IN VARIOUS WAYS,* AND INFECTED ONE HOUR LATER WITH *Str. agalactiae* (A4)

Dose mg.	*Expt. No.*	*Sulphanil-amide*	*Sulphanil-amide E.O.S.*	*Sulphone*	*Sulphone E.O.S.*	*Solusep-tasine*	*Sulphanil-amide L.S.F.*	*Sulpha-pyridine*	*Solu-pyridine*	*Average survival time of all mice receiving each dose level*	*Controls*
1	59	66	98·8	115·2	120·0	80·0	40·0	61·0	101·0		
1	61	35·2	98·8	120·0	66·0	63·0	18·8	40·0	98·8		
1	63	120·0	120·0	120·0	120·0	120·0	98·8	120·0	120·0		
1	65	80·0	44·8	49·6	120·0	98·8	58·8	42·4	82·4		
	Average	75·3	90·6	102·2	106·5	90·6	54·1	65·8	100·5	85·5	
0·5	59	16·4	26·0	84·8	120·0	52·0	14·0	37·6	80·0		16·4
0·5	61	23·6	37·6	98·8	115·2	98·8	18·8	26·0	30·8		16·4
0·5	63	101·2	120·0	120·0	108·4	120·0	61·2	120·0	84·8		42·4
0·5	65	23·6	58·8	98·8	101·2	18·8	21·2	56·4	58·8		14·0
	Average	41·2	60·65	100·6	111·4	72·4	28·8	60·0	63·6	67·3	22·3
0·25	59	30·4	21·2	103·6	103·6	18·8	18·8	23·6	42·4		
0·25	61	35·2	37·6	56·4	120·0	40·0	14·0	14·0	35·2		
0·25	63	120·0	101·2	120·0	120·0	77·6	63·6	23·6	120·0		
0·25	65	16·4	16·4	77·6	87·2	56·4	14·0	37·6	16·4		
	Average	50·5	44·1	89·4	107·7	48·2	27·6	24·7	53·5	55·7	
Average all doses		55·6	65·1	97·0	105·5	70·4	36·8	50·1	72·5		

* The doses of solubilised compounds were adjusted so that identical amounts of the active drug were given.

TABLE II

THE SURVIVAL TIME IN HOURS OF MICE GIVEN SULPHANILAMIDE AND SULPHONE, SUBCUTANEOUSLY, AS THE FREE DRUG AND SOLUBILISED IN VARIOUS WAYS,* AND INFECTED ONE HOUR LATER WITH *Str. agalactiae*

Dose mg.	*Exp.*	*Sulphone*	*Sulphone E.O.S.*	*Sulphanil-amide*	*Sulphanil-amide E O S*	*Solusep-tasine*	*Sulphanil-amide L.S.F.*	*Average survival time of all mice receiving each dose level*	*Controls*
2	51	120·0	120·0	103·6	49·6	98·8	35·2		
2	54	120·0	120·0	120·0	120·0	98·8	98·8		
	Average	120·0	120·0	111·8	84·8	98·8	67·0	100·4	
1	51	120·0	120·0	66·0	27·6	103·6	37·6		
1	52	120·0	101·2	77·6	35·2	23·6	35·2		
1	54	120·0	120·0	103·6	120·0	120·0	98·8		
1	57	98·8	108·4	63·6	28·4	33·2	26·0		
	Average	114·7	112·4	77·7	52·8	70·1	49·4	79·5	
0·5	51	120·0	120·0	47·2	42·5	58·8	35·2		21·2
0·5	52	80·0	113·2	16·4	35·6	23·6	16·4		35·2
0·5	54	82·4	120·0	98·8	80·0	120·0	26·0		35·2
0·5	57	73·2	98·8	37·6	18·8	28·4	26·6		14·0
	Average	88·9	113·0	50·0	44·2	57·7	26·0	63·4	26·4
0·25	52	37·6	45·2	37·6	35·2	16·4	14·0		
0·25	54	50·8	120·0	58·8	77·6	63·6	42·8		
0·25	57	38·0	120·0	23·6	35·2	40·4	16·4		
	Average	42·1	95·1	40·0	49·3	40·1	24·4	48·2	
Average of all doses		91·4	110·1	70·1	57·7	66·7	41·7		26·4

* The doses of solubilised compounds were adjusted so that identical amounts of the active drug were given.

inactive at the dose used. On the other hand, sulphanilamide E.O.S. and sulphone E.O.S. were only slightly less active than their parent compounds. These results indicate that the E.O.S. complex dissociates fairly completely in the chick-embryo, but the L.S.F. complex is very stable. If the effects of giving drugs to mice orally and subcutaneously are compared it will be seen that all the soluble derivates except sulphanilamide L.S.F. were more active than their parent compounds when given orally, and less active when given subcutaneously. Sulphone and sulphone E.O.S. provide another exception when given subcutaneously.

It is probable that all the complex molecules are more rapidly absorbed from a subcutaneous deposit than their parent compounds. We know that sulphanilamide and sulphone E.O.S. are more rapidly excreted than the parent compounds (Francis, 1946b), and the same

TABLE III

THE EFFECT OF GIVING SULPHACETAMIDE, SULPHONE AND SULPANILAMIDE, AND VARIOUS SOLUBLE DERIVATIVES OF THESE SUBSTANCES, INTO THE ALLANTOIC CAVITY OF CHICK-EMBRYOS INOCULATED ON TO THE CHORIO-ALLANTOIS WITH *Str. agalactiae*. SIX EGGS PER GROUP IN EACH OF SIX EXPERIMENTS

	Sulphone	*Sulphone E.O.S.*	*Controls*	*Sulphanil-amide*	*Sulphanil-amide E.O.S.*	*Sulphanil-amide L.S.F.*	*Sulpha-cetamide*	*Controls*
Dose, mg.	2·5	2·5	—	6·6	6·6	6·6	6·6	—
Mean specific death rate ...	1·5	1·7	2·6	1·2	1·5	2·6	2·2	2·2
Mean non-specific death rate	1·8	1·3	0·39	1·2	1·6	0·5	1·0	0 5

appears to be true of solupyridine and perhaps sulphanilamide L.S.F. This would account for the lower activity of the soluble complexes when given subcutaneously, although the low potency of sulphanilamide L.S.F. might also be explained by the lack of dissociation. Sulphone is very insoluble and it appears that the doses we used did not produce an effective blood-level, but sulphone E.O.S. was the most potent of the complex molecules.

The complex molecules are probably not absorbed as such from the intestine, and absorption is delayed. A blood-level is therefore obtained for a longer period, which renders the drugs more potent. Again, however, it appears that sulphanilamide L.S.F. is so stable that it is only partially absorbed.

It will be noted that the relative crude potency of sulphanilamide/sulphone was 1 : 7·06, which gives an intrinsic potency of 1 : 3·5, whereas that obtained by Francis and Peters (1947) was 1 : 2·5. It is probable that the true value is 1 : 3, which is the same as that obtained in the chick-embryo (Francis, Peters and Davies), (1947).

TABLE IV

THE RELATIVE CRUDE POTENCIES OF SULPHANILAMIDE, SULPHAPYRIDINE AND SULPHONE, TOGETHER WITH VARIOUS SOLUBLE DERIVATIVES, GIVEN PER OS, AND SUBCUTANEOUSLY, ONE HOUR AFTER THE INTRAPERITONEAL INOCULATION OF 0·5 C.C. OF A 22-HOUR BLOOD-BROTH CULTURE OF *Str. agalactiae*

Drug	*Drug given per os*	*Drug given subcutaneously*
Sulphanilamide	1·0	1·0
Sulphanilamide E.O.S. ...	1·6	0·61
Soluseptasine	2·27	0·88
Sulphanilamide L.S.F. ...	0·65	0·32
Sulphapyridine	1·06	—
Solupyridine...	2·45	—
Sulphone	7·06	2·38
Sulphone E.O.S.	12·90	5·06

Again, the relative crude potency of sulphanilamide E.O.S./sulphone E.O.S. when given orally or subcutaneously was about 1 : 8. In the two series of chick-embryo experiments in this study, although the death rate in the controls of the sulphone series was greater than in the sulphanilamide series, a dose of 2·5 mg. sulphone gave nearly the same protection as an average dose of 6·6 mg. of sulphanilamide. In this study sulphapyridine only had the same crude potency as sulphanilamide in the mouse, giving it an intrinsic potency of 0·6, compared with 1·25 in the previous study, the true value probably being 1·0. Soluseptasine and solupyridine also had the same crude potency in the mouse.

All the foregoing experiments indicate that sulphone is the only one of the "sulphonamides" which is markedly more potent than sulphanilamide against *Str. agalactiae*. It has been shown by Francis (1946b) that it is the most persistent of the sulphonamides when injected into the cow's udder, being 10 to 20 times more persistent than sulphanilamide. It therefore appears that it will be the sulphonamide of choice for injection into the udder. A satisfactory suspension in liquid paraffin can be dispensed in collapsible tubes, and it causes less disturbance in the udder than the E.O.S. derivative. In contrast to sulphone suspension the E.O.S. derivative is rapidly absorbed from the dry udder (Francis, 1946b), so there is little or no reason for using the E.O.S. derivative.

Sulphone produces better blood-levels than sulphanilamide, but unfortunately the milk-levels are lower than the blood-levels (Francis ; 1946a, 1946b). The drug may, however, have a place in the treatment of mastitis by oral dosing as it is less toxic to the cow than is sulphanilamide. In man and most other species it is more toxic than sulphanilamide.

SUMMARY

The relative potencies of sulphanilamide, sulphapyridine, 4 : 4′-diaminodiphenyl sulphone and various soluble derivatives of these compounds were compared in the chick-embryo and mouse.

The results with the free compounds have, in general, confirmed those obtained previously and are summarised in Tables III and IV.

With the exception of sulphanilamide L.S.F., which was of very low activity, the soluble derivatives had about the activity one would expect from the amount of active drug they contain. The results, however, varied somewhat with the route of administration. These differences, together with the application of the findings to the treatment of mastitis, are briefly discussed.

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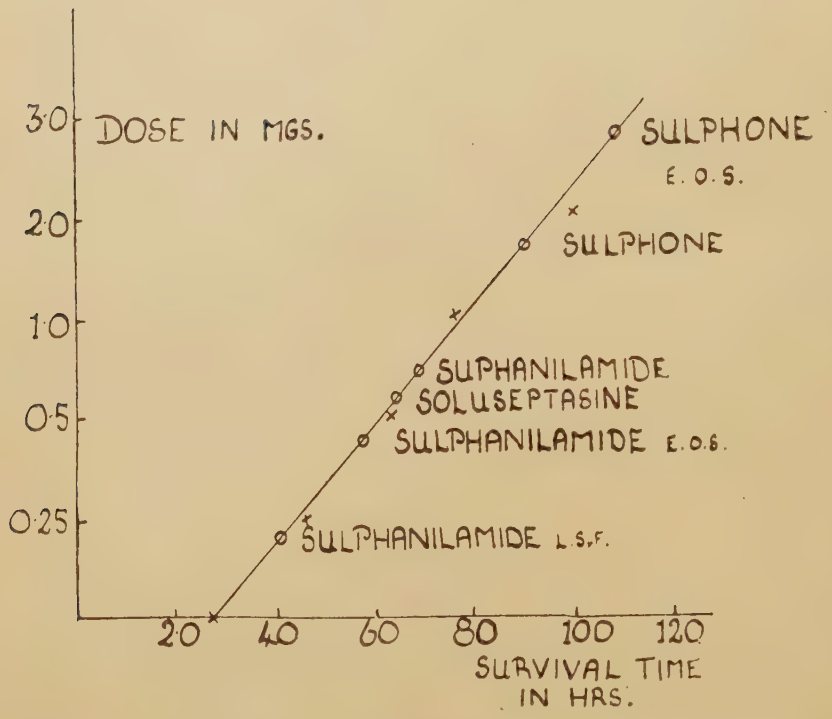

FIG. 1.

The survival time of mice given sulphanilamide, sulphone and sulphapyridine per os as the free drug and solubilised in various ways, and infected one hour later with *Str. agalactiae* (A4). (Table I.)

The curve is obtained by averaging the survival time of all mice inoculated with each dose level (x); the survival time of all mice receiving each drug is then averaged and placed on this curve (o).

The relative activity can be calculated from the vertical distance between any two drugs.

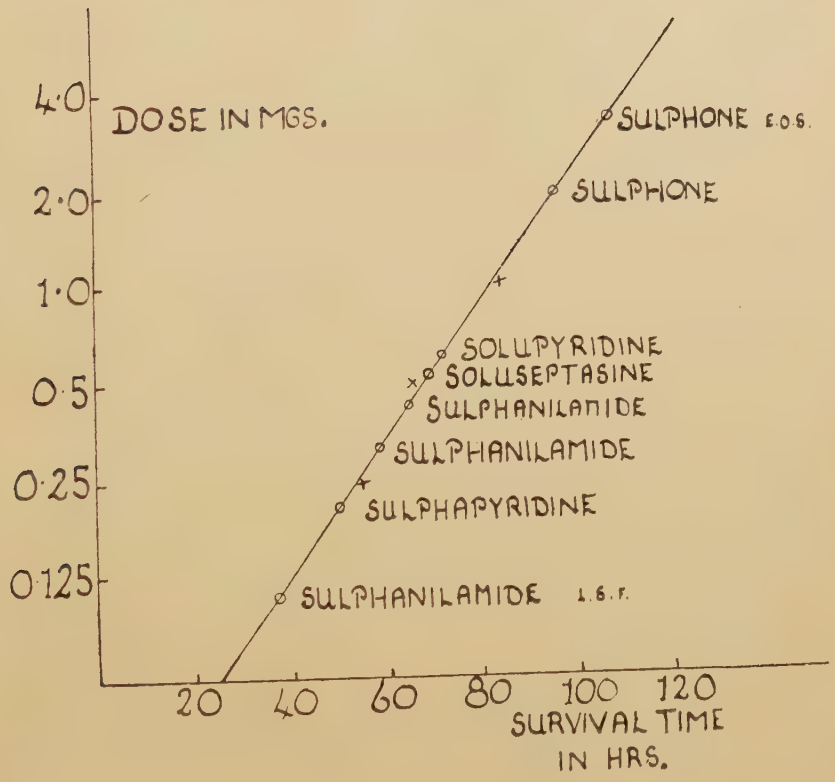

FIG. 2.

The survival time of mice given sulphanilamide and sulphone, subcutaneously, as the free drug and solubilised in various ways, and infected one hour later with *Str. agalactiae* (A4). (Table II.)

STUDIES ON *STREPTOCOCCUS AGALACTIAE* INFECTION IV.—ATTEMPTS TO PRODUCE IMMUNITY IN THE MOUSE

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INTRODUCTION

Vaccines have been used extensively in attempts to control mastitis associated with *Str. agalactiae*. The extensive literature has been reviewed by Munch-Petersen (1938) and it need only be said here that the results have generally been unsatisfactory. By far the most comprehensive experiments were those of Seelemann who concluded that vaccines were useless for prevention or cure.

The efficacy of nearly all vaccines for human use are judged by experiments in laboratory animals and there is much evidence that if a vaccine produces no immunity against an experimental infection in laboratory animals, it will produce no immunity against the natural infection in man and vice versa. The production of a mouse-virulent strain of *Str. agalactiae* (Francis and Peters, 1947) provided an opportunity to carry out critical immunological experiments with this organism.

EXPERIMENTAL

In order to provide the most favourable conditions for the production of immunity, in most experiments live cultures of the mouse-avirulent strain of *Str. agalactiae* (A4) were inoculated subcutaneously. The mouse-virulent strain of *Str. agalactiae* (A4) was inoculated intraperitoneally to test immunity. In all experiments comparable groups of mice were selected ; groups were then vaccinated at intervals so that the test dose was given to the controls and all vaccinated groups at the same time. The detailed results are shown in Table I.

Experiment 1/58: 0·4 ml. of a 24-hour serum-broth culture was used for vaccination, and immunity was tested eight to 15 days later. Only three of nine control mice died but even against this mild infection no immunity was produced.

Experiment 1/68: The procedure was similar to that in the previous experiment, except that the mice in one group were vaccinated with 5 ml. of culture* None of the controls survived and only one in each of two vaccinated groups.

Experiment 2/23: Bazeley (1940, 1942) showed that young heat-killed cultures of *Str. equi*, which are capsulated, produce a much better immunity than old cultures both in laboratory animals and in horses. An 18-hour culture of the avirulent strain was there-

* When these large doses were used the culture was centrifuged and the deposit suspended in an appropriate amount of broth.

fore inocculated into ten times its volume of warm broth containing 10 per cent. serum and 1 per cent. glucose and incubated for

TABLE I

THE RESULTS OF INJECTING MICE INTRAPERITONEALLY WITH THE MOUSE-VIRULENT STRAIN OF *Str. agalactiae* (A.4) AFTER VACCINATION IN VARIOUS WAYS WITH THE MOUSE-AVIRULENT STRAIN

Expt.	Vaccine: Age and viability of culture	Vaccine: Dose and suspending medium	Interval between vaccine and test-dose in days	Volume of test-dose, mls. culture	No. of mice	No. of deaths* 1st day	No. of deaths* 2nd day	Survivors
1/58	24-hour serum broth	0·4 ml., broth	15	0·4	6	2	2	2
			8	0·4	9	1	3	5
	Controls			0·4	9	3	–	6
1/68	24-hour serum broth	5 ml., broth	14	0·7	10	9	–	1
		0·5 ml., „	14	0·7	10	10	–	–
		5 ml. „	7	0·7	10	10	–	–
		0·5 ml., „	7	0·7	10	9	–	1
	Controls			0·7	10	10	–	–
2/23	Four-hour glucose-serum broth — Live ...	1 ml., broth	42	0·5	7	4	2	1
		5 „ „	42	0·5	9	7	–	2
	Four-hour glucose-serum broth — Heated 56°C. —30 min.	1 „ „	42	0·5	15	15	–	–
		5 „ „	42	0·5	15	14	–	1
	Four-hour glucose-serum broth — Live ...	1 „ „	21	0·5	11	10	1	–
		5 „ „	21	0·5	12	11	–	1
	Four-hour glucose-serum broth — Heated 56°C. —30 min.	1 „ „	21	0·5	14	12	2	–
		5 „ „	21	0·5	15	15	–	–
	Controls			0·5	14	13	–	1
				0·25	14	10	3	1
				0·125	14	9	4	1
2/29	24-hour serum broth — Live ...	1 ml., broth	35	0·7	19	16	–	3
		1 „ oil	35	0·7	17	15	–	2
		5 „ „	35	0·7	19	16	–	3
	24-hour serum broth — Heated 56°C. —20 min.	5 „ „	35	0·7	14	14	–	–
	24-hour serum broth — Live ...	1 „ broth	23	0·7	19	19	–	–
		1 „ oil	23	0·7	18	15	–	3
		5 „ „	23	0·7	18	16	–	2
	24-hour serum broth — Heated 56°C. —20 min.	5 „ „	23	0·7	16	15	–	1
	Controls			0·7	19	19	–	–
				0·35	16	8	2	6
				0·175	17	12	–	5

* The mice were observed for five days but no deaths occurred after the second day.

four hours. Capsulated organisms have never been observed in these cultures, neither are they more virulent for mice than ordinary 18-hour cultures (Francis and Peters, 1947), but they were used for vaccinating mice in doses of 1 and 5 ml. Some groups of mice were inoculated with cultures which had been killed by heating at 60° C. for 30 minutes. As no immunity had been demonstrated in earlier experiments up to 14 days after vaccination, on this occasion the virulent strain was injected 21 and 42 days after vaccination ; no immunity was demonstrated in any group.

Experiment 2/29: An 18-hour serum-broth culture, alive and killed by heating at 56° C. for 20 minutes respectively, suspended in saline or in an oil-lanolin mixture,† was used for vaccination in 1 ml. and 5 ml. amounts. The virulent strain was injected 23 and 35 days after vaccination. There were a few survivors in some of the vaccinated groups but their numbers were less than in a control group inoculated with half the test dose, so it can be assumed that no serviceable degree of immunity was produced.

DISCUSSION

It may be felt that the acute streptococcal infection of mice is very different from the chronic infection which often characterises mastitis in cattle. Nevertheless the infection in mice is readily controlled by the sulphonamides (Francis, Peters and Davies, 1947) and it is reasonable to suppose, particularly as a local abscess is often produced, that if *Str. agalactiae* were capable of stimulating immunity this would have been demonstrated.

The general failure of vaccines to control mastitis has already been mentioned and the experiments reported in this paper provide no evidence which would encourage further attempts to produce immunity in cattle. Indeed, the natural course of the disease, in which all four quarters may be successively infected at variable intervals, suggests that infection does not produce an effective immunity.

SUMMARY

The mouse-avirulent strain of *Str. agalactiae* (A4) was inoculated subcutaneously in an attempt to vaccinate mice, and immunity was tested by the intraperitoneal injection of the mouse-virulent strain.

Cultures of varying ages were used for vaccination, the vaccinating dose was sometimes suspended in oil, and immunity was tested at periods varying from seven to 42 days. There was no evidence that resistance had been increased, despite the fact that the vaccinating dose often produced a small abscess which had usually healed before the test dose was given.

† 10 grammes lanolin in 50 c.c. of arachis oil.

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